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THE SULPHONAMIDES: RELATIVE POTENCIES AND SPECIFICITY OF ACTION

BY

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(Received August 2, 1951)

Woods showed that *p*-aminobenzoic acid antagonized the antibacterial action of the sulphonamides in a competitive manner. He suggested that *p*-aminobenzoic acid was an essential metabolite for bacteria and that the sulphonamides acted by interfering with this metabolite. It has since been shown that *p*-aminobenzoic acid is an essential metabolite for bacteria (see Woods and Nimmo-Smith, 1949) and the view that the sulphonamides act by interfering with the utilization of *p*-aminobenzoic acid has come to be generally accepted. An extension of this theory is that the action of the sulphonamides is " non-specific " and that the sulphonamides have the same relative potencies against different organisms (Hawking and Stewart Lawrence, 1950). These authors use the expression, " sulphanilamide coefficient," to depict the relative activity of a sulphonamide, as though it can be used as a general term that can be applied to the relative potency of a drug against any organism.

The concept of the " non-specificity " of action of the sulphonamides has been able to grow up and persist because most people have only studied the action of one or of a very few sulphonamides against a variety of organisms, or of a large number of sulphonamides against one or a very few organisms. In particular both streptococci and sulphanilimidé have not been included in many recent studies. No single paper has been found in which the relative potencies of all the common sulphonamides have been compared *in vitro* against a number of pathogens. It is the purpose of this paper to present such information and to show that there are large differences in the relative potencies of the sulphonamides against different organisms. Similar data on the relative blood-concentrations produced by the sulphonamides in a variety of species have been given by Francis (1949).

METHODS

Falling threefold dilutions of the drugs were prepared, the concentration in the first tube being 1/100. These dilutions were added in 0.2 ml. amounts to 1.8 ml. of medium containing agar, to give slopes containing 1/1,000, 1/3,000, etc., of the drugs. " Lab-Lemco " was used as a basis of the medium for the experiments shown in Table I and beef infusion for those shown in Table II. Evans's peptone was used and horse blood, as it has been shown that horse blood tends to neutralize sulphonamide inhibiting substances, whereas blood from some other species does not (Harper and Cawston, 1945). The blood was used at a concentration of 5 per cent (v/v) and 1 per cent (w/v) glucose was also added to ensure that all organisms would grow well. The agar was

maintained at 70 to 80° C. during distribution, which led to the production of "chocolate" agar. A small series of experiments was carried out in milk containing 1 per cent (w/v) glucose; bromcresol-purple was present in the medium and the results were judged by changes in the colour of the indicator. The results are included in Table I. Broth cultures which were incubated overnight were used for inoculating all media, a loopful of these cultures being streaked on to the slopes or inoculated into the milk.

The experiments shown in Table I were carried out with one batch of Lemco medium and all organisms were included in each of three consecutive tests. In the first series of three tests shown in Table II one batch of medium was used and it was sown with *Str. pyogenes, Str. pneumoniae, Past. septica,* and *Bact. coli.* In a second series of three tests the medium was sown with *Salm. cholera suis, N. gonorrhoea, N. meningitidis,* together with *Str. pneumoniae* to act as a link with the first series. The growths in each tube were recorded as 0, 1, 2, or 3, and similar endpoints were obtained with the respective drugs and organisms in each of the three experiments in the two series. The average growth in tubes 1, 2, etc., in the three tests of each series was calculated. A curve was constructed for each drug over the critical range and the average concentration giving 50 per cent growth found by interpolation. This concentration was expressed in "tube numbers." All averages were then worked out on the basis of "tube numbers," i.e., a log. scale was used and these averages were converted to the actual dilutions shown in Tables I and II. Thus, if 50 per cent growth occurred in tube 3, the concentration would be 1/9,000 (dilution in first tube 1/1,000), whereas if the average result were 3.5 the concentration would be 1/15,590. It will be seen that the average 50 per cent endpoint with all drugs, against *Str. pneumoniae,* was at a dilution of 1/217,700 in the first series of experiments and 1/243,000 in the second. There was just over a twofold difference in the sulphanilamide coefficient with some drugs, but this is less than the threefold difference between two consecutive tubes. These findings indicate the degree of variation and that all the results in Table II can be considered together.

Results

In the following account 4 : 4′diaminodiphenyl sulphone is referred to as sulphone and is included under the general term "sulphonamide." It is not, of course, a sulphonamide, but its action is strongly antagonized by *p*-aminobenzoic acid.

The results in Table II are considered first because they are felt to be more in accord with those obtained by other workers. It will be seen that the organisms can de divided into two distinct groups on the basis of the average "sulphanilamide coefficient" of the heterocyclic drugs obtained when they are used. With streptococci, pneumococci, and gonococci the coefficient varied from 2.3 to 12.6, but with the other organisms from 35 to 139. The compounds can be divided into the non-heterocyclic sulphonamides (sulphanilamide, sulphaguanidine, and sulphone), and the remainder, which are heterocyclic. Sulphone was more active than any other compound against *Str. pneumoniae,* and against *Str. pyogenes,* with the exception of sulphathiazole, which gave particularly good results in this series of experiments. Against all other organisms the non-heterocyclic sulphonamides were less active than the heterocyclic.

If only the heterocyclic sulphonamides are considered it will be noted that they were nearly always placed in the same order of activity : the order shown in Tables I and II, sulphapyridine being the least potent and sulphathiazole the most potent. The exceptions were sulphamezathine against *Str. pyogenes* and sulphadiazine against

TABLE I

THE INHIBITORY ACTION OF THE SULPHONAMIDES *in vitro*: AVERAGE RESULTS OF THREE CONSECUTIVE TESTS

Organism		Dilutions giving 50% inhibition									Sulphanilamide coefficient (A)							
		Sulphanilamide	Sulphaguanidine	Sulphone	Sulphapyridine	Sulphamezathine	Sulphamerazine	Sulphadiazine	Sulphathiazole	Logarithmic average	Sulphaguanidine	Sulphone	Sulphapyridine	Sulphamezathine	Sulphamerazine	Sulphadiazine	Sulphathiazole	Average of heterocyclic sulphonamides (B)
Str. pyogenes (Lancefield Group A)	Tube No.	3.5	3.7	4.7	3.5	3.5	3.9	4.7	4.7	4.0	1.2	3.7	1.0	1.0	1.6	3.7	3.7	2.2
	Dilutions†	15,600	19,400	58,200	15,600	15,600	25,200	58,200	58,200	27,000								
Str. pneumoniae (7465)*	Tube No.	2.9		5.6	4.8	4.0	4.3	4.3	3.5	4.2		19.3	8.0	3.3	4.6	4.6	1.9	4.5
	Dilutions	8,100		156,500	65,000	27,000	37,500	56,600	33,600									
Past. septica (D.B.183)	Tube No.	3.4	4.7	4.6	6.1	6.8	7.4	8.4	6.5	6.0	4.2	3.7	19.4	41.8	77.1	242.3	30.1	82.1
	Dilutions	14,000	58,200	52,200	271,100	585,100	1,080,000	420,800	243,000									
Salm. cholerae suis (Kunzendorf)	Tube No.	1.2	1.7	2.9	3.9	4.5	5.0	5.7	5.6	3.8	1.8	6.8	19.1	38.8	67.5	145.6	109.3	76.1
	Dilutions	1,200	2,200	8,100	22,900	46,700	81,000	174,700	156,600	21,700	(1.7)	(3.4)	(15.6)	(14.0)	(41.9)	(140.3)	(72.6)(C)	
Salm. dublin	Tube No.	0	0.4	0.9	1.9	2.2	2.5	3.2	3.0	1.7	3.0	8.0	25.0	37.0	52.0	106.0	90.0	62.0
	Dilutions	100	300	800	2,500	3,700	5,200	10,600	9,000	2,200	(2.4)	(1.7)	(11.2)	(14.0)	(14.0)	(52.2)	(52.2)(C)	
Logarithmic average	Tube No.	2.2	2.6	3.7	4.0	4.2	4.6	5.2	4.6	3.9	1.6	5.2	7.2	8.9	13.9	26.9	13.9(D)	
	Dilutions	3,747	5,799	19,420	27,000	33,620	52,190	100,900	52,190	25,200								

* National Collection of Type Cultures.

† The denominators of dilutions are shown.

A. The sulphanilamide coefficient is obtained by dividing the denominator of the dilution giving 50% inhibition with sulphanilamide by the denominator of the dilution of any other drug giving 50% inhibition.

B. The average sulphanilamide coefficient of the heterocyclic sulphonamides is obtained by averaging the individual coefficients.

C. Figures in brackets represent the average sulphanilamide coefficients in four series of tests in which the organisms were grown in milk containing 1% glucose: bromcresol-purple was present in the medium and the results were judged by changes in the colour of the indicator.

D. The average sulphanilamide coefficients on the bottom row are obtained from the logarithmic averages.

TABLE II

THE INHIBITORY ACTION OF THE SULPHONAMIDES *in vitro*: AVERAGE RESULTS OF THREE CONSECUTIVE TESTS

Organism		Dilutions giving 50% inhibition									Sulphanilamide coefficient (A)							
		Sulphanilamide	Sulphaguanidine	Sulphone	Sulphapyridine	Sulphamezathine	Sulphamerazine	Sulphadiazine	Sulphathiazole	Logarithmic average	Sulphaguanidine	Sulphone	Sulphapyridine	Sulphamezathine	Sulphamerazine	Sulphadiazine	Sulphathiazole	Average of heterocyclic sulphonamides (B)
Str. pyogenes (Lancefield Group A)	Tube No.	4.3	4.4	5.6	4.4	3.9	4.3	5.1	6.0	4.8	1.1	4.2	1.1	0.6	1.0	2.4	6.5	2.3
	Dilutions†	37,530	41,890	156,600	41,890	24,180	37,530	90,380	243,000	61,530								
Str. pneumoniae (7465)*	Tube No.	4.4	5.6	7.2	5.3	5.7	5.9	6.1	7.0	5.9	3.7	21.6	2.7	4.2	5.2	6.6	17.4	7.2
	Dilutions	41,890	156,600	907,800	112,600	174,700	217,700	277,100	729,000	217,700								
Str. pneumoniae (7465)	Tube No.	4.2	5.5	7.5	5.7	6.3	5.8	5.5	7.5	6.0	4.1	37.6	5.2	10.1	5.8	4.1	37.6	12.6
	Dilutions	33,620	140,300	1,263,000	174,800	337,800	195,000	140,300	1,263,000	243,000								
Neisseria gonorrhoea (6820)	Tube No.	2.8	3.5	3.8	4.3	4.3	4.5	5.4	5.0	4.2	2.2	3.0	5.3	5.3	6.5	17.4	11.2	9.1
	Dilutions	7,224	15,590	21,670	37,530	37,530	46,770	125,700	81,000	33,620								
Neisseria meningitidis (3372)	Tube No.	3.0	4.3	5.1	5.5	5.4	6.4	7.4	8.0	5.6	4.2	10.1	15.6	13.9	42.0	125.7	243	88.0
	Dilutions	9,000	37,530	90,380	140,300	125,700	377,700	1,131,000	2,187,000	163,600								
Past. septica (D.B. 183)	Tube No.	2.4	3.2	3.4	4.0	5.0	5.2	5.9	6.4	4.4	2.4	3.0	5.8	17.4	21.7	46.8	80.9	34.5
	Dilutions	4,655	11,210	13,960	27,000	81,000	100,900	217,700	377,000	43,770								
S. cholerae suis (Kunzendorf)	Tube No.	2.7	3.5	4.4	6.2	6.2	7.3	7.4	7.8	5.7	2.4	6.5	46.8	46.8	156.6	175.0	271.0	139.2
	Dilutions	6,473	15,590	41,890	302,600	302,600	1,014,000	1,131,000	1,755,000	172,800								
B. coli (Evans 235)	Tube No.	2.9	2.7	4.0	4.9	5.5	6.5	6.5	6.5	4 9	0.8	3.3	9.1	17.4	52.2	52.2	52.2	36.6
	Dilutions	8,063	6,473	27,000	73,620	140,300	420,800	420,800	420,800	75,820								
Logarithmic average	Tube No.	3.3	4.1	5.1	5.0	5.3	5.7	6.2	6 8	5.2	2.3	6.9	6.2	8.6	13.4	23.1	44.8 (C)	
	Dilutions	13,070	30,130	90,380	81,000	112,600	174,800	302,600	585,100	100,900								

* National Collection of Type Cultures.

† The denominators of dilutions are shown.

A. The sulphanilamide coefficient is obtained by dividing the denominator of the dilution giving 50% inhibition with sulphanilamide by the denominator of the dilution of any other drug giving 50% inhibition.

B. The average sulphanilamide coefficient of the heterocyclic sulphonamides is obtained by averaging the individual coefficients.

C. The average sulphanilamide coefficients in the bottom row are obtained from the logarithmic averages.

N. gonorrhoea. There was no difference in the action of sulphamerazine, sulphadiazine, and sulphathiazole against *Bact. coli,* whereas there was a fivefold difference with some other organisms.

A comparison of Tables I and II shows that there were big changes in the order of potency when " Lab-Lemco " was used as a basis for the medium instead of beef infusion. The most striking difference between the two series is that sulphathiazole was much less potent in the first series than in the second. This difference was so striking that the stock solution of sulphathiazole, which had been in the refrigerator since the earlier series of tests, was included on two occasions in the second series ; it was as active as the current stock solution of sulphathiazole. It would appear that there was some difference between the media in the two series of experiments that specifically influenced the action of sulphathiazole. The sulphanilamide coefficients, except with sulphathiazole, were much larger against *Past. septica* in the first series of experiments than in the second. It will be seen that the sulphanilamide coefficients obtained in milk (Table I) were consistently lower than in the chocolate agar medium with " Lab-Lemco " as a basis.

It has been shown that the results obtained with the different organisms shown in Table II may be compared, and it will be seen that sulphathiazole was about seven times as active against *Salm. cholerae suis* as against *Str. pyogenes,* but sulphanilamide was about a fifth as active against the former as against the latter organism. As already stated, there is little difference in the relative potencies of the different sulphonamides against streptococci, but the action of the heterocyclic sulphonamides is much better than that of the non-heterocyclic sulphonamides against some of the Gram-negative organisms.

Discussion

The results reported in this paper are supported by those of other workers. Thus Lauger, Suter, and Martin (1944) found that the action of one sulphonamide on a particular micro-organism does not guarantee its action on another. Compounds tested against pneumococci, streptococci, and *Bact. coli* may inhibit one, two, or all three of the micro-organisms. McIntosh and Dyrsdale (1945) described a meningococcus that was sensitive to sulphamezathine and sulphathiazole but not to sulphapyridine. Recently, Schweinburg and Rutenburg (1949) have described six different cases of bacterial infections that proved susceptible to one sulphonamide after failing to respond to another. The bacteria were shown to be susceptible to the effective sulphonamide but not to the others. Schmidt, Sesler, and Hughes (1944) found the sulphapyrimidine drugs to be about equally active against streptococci and Friedländer's bacillus, *Bact. coli,* and shigella species. On the other hand, sulphamezathine was twice as active as sulphadiazine or sulphamerazine against pneumococci and staphylococci.

Wyss, Grubaugh, and Schmelkes (1942) and Havinga, Julius, Veldstra, and Winkler (1946) obtained different relative potencies against the same organism in different media, and a large number of results collected from the literature by Stewart Lawrence and Francis (1952) show that the reported sulphanilamide coefficients of sulphathiazole against *Bact. coli* vary from 25 to 250 with a mean of 53.0, which is very close to the mean of 52.2 obtained in the present study. It should perhaps be stated here that although the figure of 50 is often quoted as the sulphanil-

amide coefficient of sulphathiazole the maximum coefficient observed *in vivo*, after corrections have been made for differences in blood concentrations, is only 13.3.

Schmidt and Sesler (1946) pointed out that the evidence supporting the " non-specific " action of the sulphonamides had never been very conclusive. They showed that with the sulphanilanilides the antagonistic action of *p*-aminobenzoic acid could be demonstrated with *Bact. coli* and Friedländer's bacillus, but not with pneumococci. Like sulphones, the sulphanilanilides are much more effective against Gram-positive than against Gram-negative organisms. Sulphadiazine was only twice as active as sulphanilamide against pneumococci *in vitro*, but 32 times as active against Friedlander's bacillus. Tamura (1944) showed that *p*-aminobenzoic acid did not inhibit the bacteriostatic action of the sulphonamides against *B. tularense*.

Recent work on the mode of action of the sulphonamides has been reviewed by Dible and MacLennan (1951). It is now known that *p*-aminobenzoic acid is concerned in the metabolism of folic acid, and it seems probable that the action of the sulphonamide is primarily directed against the metabolic conversion of *p*-aminobenzoic acid into pteroyl-glutamic acid or possibly into pteroic acid. The fact that two drugs are inhibited by *p*-aminobenzoic acid does not prove that they act in the same way. Thus, the action of a drug as dissimilar from the sulphonamides as proguanil may be inhibited by *p*-aminobenzoic acid (Thurston, 1950), and Yegian and Long (1951) showed that, although *p*-aminosalicylic acid and the sulphonamides are both inhibited by *p*-aminobenzoic acid, tubercle bacilli made resistant to *p*-aminosalicylic acid are not resistant to the sulphonamides or sulphone. They indicate that there may be similar specific differences between the sulphonamides, and this is known to be true (Schweinburg and Rutenburg, 1949).

The position has become very complex, but it may eventually be possible to explain the various specificities in the actions of the sulphonamides. In the meantime it appears that such simple conclusions as those put forward by Bell and Roblin (1942) are not tenable. They state that the more negative the SO_2 group of an N^1-substituted sulphanilamide derivative, the greater is its bacteriostatic potency, which leads them to state that, if the relative electron attracting power of the N^1-substituent is known, it is possible for the first time to predict the bacteriostatic potency of any new sulphanilamide derivative of this type. This is probably true against *Bact. coli* in a defined medium but not against all organisms in that medium or even against one organism in different media.

Summary

1. The relative potencies of the common sulphonamides and 4 : 4'diaminodiphenyl sulphone (sulphone) were compared *in vitro* against a number of pathogens.

2. In the most satisfactory series of experiments (Table II) the average sulphanilamide coefficient of the heterocyclic sulphonamides was only 2.3 against streptococci but 139 against *Salm. cholerae suis* and 36 against *Bact. coli*.

3. Sulphone was more active than any of the sulphonamides, except sometimes sulphathiazole, against streptococci, but it was less active than the heterocyclic sulphonamides against Gram-negative organisms.

4. The significance of these findings is discussed.

I am indebted to Mr. F. Vernon for his assistance in this work.

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THE EVALUATION OF ANALGESIC POTENCY OF DRUGS USING THERMAL STIMULATION IN THE RAT

BY

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(Received August 9, 1951)

Numerous methods have been devised for the application of noxious stimuli to a variety of experimental animals in an endeavour to find a satisfactory method of estimating the analgesic potency of drugs. The stimulus applied produces a protective response of some kind which is interpreted as a pain response, and the effect upon it of the drug under investigation may then be studied. One may doubt the validity of interpreting the human experience of pain in terms of the objective response of an animal. The results of recent years, however, do appear to show some parallel between relative potency as estimated in animals and clinical experience in the use of drugs of the morphine group and their synthetic " analogues."

Of recent years the use of thermal stimuli in the form of radiant heat has come into general use for the investigation of analgesic phenomena in man and animals. The method devised by Hardy, Wolff, and Goodell (1940) has been much used on man by a variety of investigators; in this country attempts to use this technique have not met with success (Dodds, Lawson, Simpson, and Williams, 1945 ; Thorp, 1946). Application of similar techniques to the rat, however, have been uniformly satisfactory. This anomaly formed the basis of the present investigations, which were undertaken in an attempt to find an explanation of the discrepancy.

When radiant heat is used there are two methods of grading the stimulus :

(1) Exposure is permitted for a fixed time interval and the intensity of the radiation varied to produce reaction just within this time. Any increase in the intensity of radiation required to produce the response under the influence of a drug is interpreted as depression of the pain sense (Thorp, 1946).

(2) The time for reaction to a fixed stimulus is measured and prolongation of this beyond the normal is taken as an indication of analgesia (D'Amour and Smith, 1941 ; Davies, Raventos, and Walpole, 1946). Such experiments are less well controlled, since the intensity of the radiant heat stimulus is not necessarily increasing proportionately with time.

No comparison can be made, by radiant heat techniques, of the normal thermal thresholds in man and the rat or of the threshold elevating effects of analgesic drugs in the two species. In the present study it was decided that temperature measurement would be a more reliable index of the response to thermal stimulation ; a direct comparison should then be possible between the interpretation of a pain response by the human subject and the objective response of the rat.

METHODS

The apparatus consists of a heating element shown in the accompanying photographs (Figs. 1 and 2). A strip of uniform oxidized " Nichrom " tape (0.25 inch width $\times$ 0.006 inch thickness) is shaped over one area so as to form an almost complete circle which is to enclose part of the rat tail. This loop lies transversely across a channel in a small board along which the tail lies. The free ends of the resistance tape are secured to the board—one permanently (A, Fig. 1), the other slotted and held down by two pieces of plastic (B and C). Lateral pressure on the extremity (F) enables the circumference of the heater loop to be varied at will, so as to fit tails of varying size. The screw (D) passes through the slotted resistance tape and enables this to be fixed in position. The resistance tape has sufficient flexibility for this purpose, whilst its thermal capacity is low so that further heating of the tail when the current

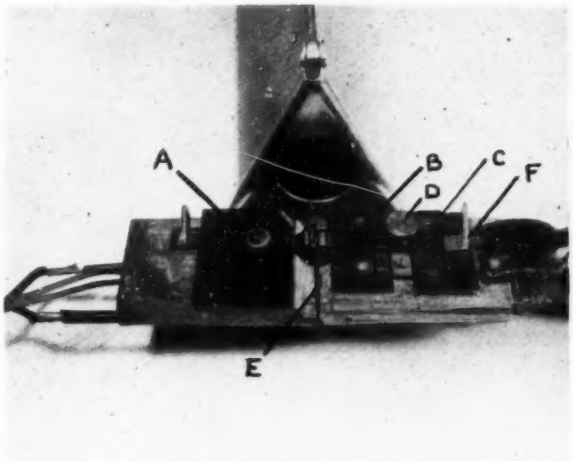

FIG. 1.—Apparatus for the application of a measured thermal stimulus to a rat tail.

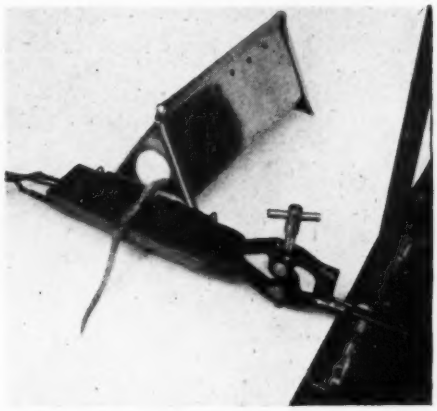

FIG. 2.—Apparatus for thermal stimulation in use; note the rat in specially designed holder.

is discontinued does not occur. Its resistance permits rapid heating with an alternating current of 5.5–6.5 amp. at 8V. Firm but not tight contact between heater and tail is essential. The temperature at which the tail reaction occurs is measured by a thermocouple of copper-constantan (E) lying transversely across the heater loop, the wires running along the tail groove. The couple must be sensitive and of small thermal capacity, otherwise low temperature readings are obtained. The wires used were of 32 gauge (0.011 inch); the ends were beaten out flat to form very thin plates which were then joined with a minimal amount of solder. A cold junction in ice was included in the circuit and readings made on a sensitive galvanometer.

When the heater is used in an experiment, it is not possible to assess the accuracy with which the temperature measurement is made. In view, however, of the results of numerous experiments carried out in man with a similar device, it is believed that the temperature readings approximate to the actual skin contact temperature applied. At first considerable difficulty was experienced in the matter of holding animals in position during heating, for it is essential that they remain quiet. Ultimately a holder was devised which has proved satisfactory (Figs. 1 and 2). In this holder ordinary Wistar strain rats may be used without previous training; the shape of the holder is such that the animal does not possess the sense of instability which cylindrical holders give and is unable to rotate along a longitudinal axis, which is a great trouble with tubular holders.

The only preliminary treatment required by the animals for the technique consists in shaving the tails over about one inch of length towards the base so as to ensure close contact between the epidermis and heater; otherwise unsatisfactory readings are obtained. This procedure is carried out the previous day so that the hypersensitivity induced settles down before an experiment is carried out.

In the weight group used (120–150 g.) the normal temperature of reaction falls within quite a narrow range (38°–40° C.) for most animals. It tends to rise somewhat in heavier animals, owing possibly to the increased thickness of the epidermis. The normal response consists of a sudden sharp twitch of the tail, which often disengages it from the grip of the heater; should any attempt be made to continue the stimulus, a struggle follows, which suggests that a pain threshold corresponds with the initial movement.

When the temperature of reaction rises under the influence of an analgesic drug, damage to the skin may occur, if heating is uncontrolled, depending upon the maximum temperature applied and the period of heating. In practice the duration of the stimulus is kept round about 10–15 seconds by means of a variable choke and the maximum temperature applied is 48° C. If no twitch occurs under these circumstances "analgesia" is assessed as complete. Response rarely occurs if this temperature is exceeded and damage is likely.

TABLE I

Scheme of testing for analgesic potency showing some results (0–100% response) for ketobemidone. For practical purposes it is unnecessary to convert the galvanometer readings to degrees centigrade. The reading "75–" indicates no reaction of the rat tail at the maximum permitted temperature (48°) and is assessed as "complete analgesia." The average normal reaction is in the range 38–40°. "75±" indicates uncertainty over the response and is always followed by further readings. Note the submaximal elevations shown by animals 3 and 5 (0.5 mg./kg.)

No. of rat	Weight (g.)	Dose		Galvanometer readings				
		mg./kg.	Volume ml.	Normal	1 min.	5 min.	10 min.	15 min.
1	129	1	0.13	60 65	75– 75–	75– 75–	75– 75–	75– 75–
2	158	1	0.16	59 58	75– 75–	75– 75–	75– 75–	75– 75–
3	157	1	0.16	59 59	75– 75–	75– 75–	75– 75–	75– 75–
4	140	1	0.14	63 62	75– 75–	75– 75–	75– 75–	75– 75–
5	152	1	0.15	61 64 59	75– 75–	75– 75–	75– 75–	75– 75–
1	186	0.75	0.19	63 64	70 71	75± 75–	75– 75–	75– 75–
2	176	0.75	0.18	64 64	75– 75–	75– 75–	75± 75– 70	75– 75–
3	180	0.75	0.18	67 65	75± 75– 75–	75± 75–	75– 75–	75– 75–
4	176	0.75	0.18	64 65	75– 75–	75± 73 70	75± 69 63	75± 69 69
5	189	0.75	0.19	63 69 68	75– 75–	75– 75–	75– 75–	75– 75–
1	134	0.5	0.13	60	64 65	74 66 67	68 66	58 59
2	138	0.5	0.14	65 56 63 60	65 65	67 68	65 65	64 65
3	154	0.5	0.15	58 58	71 73	71 72	71 75	72 73
4	133	0.5	0.13	57 60	60 61	61 64	59 60	58 61
5	130	0.5	0.13	56 57	73 74	71 72	64 67 69	64 65

In the present experiments the rats used were of an inbred Wistar strain of American origin. For each dose level, groups of 5 to 10 rats of either sex selected at random were used; each animal was used for one experiment only. They were introduced into holders, allowed to settle down for a few minutes, and control threshold levels taken to ensure that they reacted normally. The tail was next immersed in water at 45° C. for 90 seconds to dilate the tail veins and the drug under test injected in a dose volume of 0.1 ml./100 g. in about 2–3 sec. Three successive estimates of the end point were made at intervals of 1, 5, 10, and 15 min. after the injection, indiscriminate sites on the shaved area of the tail being used. By suitable timing a group of five animals can be dealt with in about thirty minutes. " Analgesia " rarely develops later than five minutes after intravenous administration of a drug. Reference to the specimen experimental Table I will clarify the procedure adopted.

RESULTS

Application of a thermal stimulus to the tail as described forms a reliable method of eliciting a motor response. Of a number of factors which might influence the normal temperature of reaction, only the initial temperature of the tail, which varies widely with room temperature, and the duration of the stimulus need be considered. A low initial temperature produces an earlier reaction temperature, so that successive readings from higher initial temperatures result in a stepwise rise of threshold figures. A relatively constant level is soon reached, however, and it is then unrelated to the initial temperature if this is above 30° C. These effects are best observed in animals under the influence of a hypnotic, since normal animals quickly grow restive with repeated stimuli (Fig. 3). The reaction temperature is independent of the duration

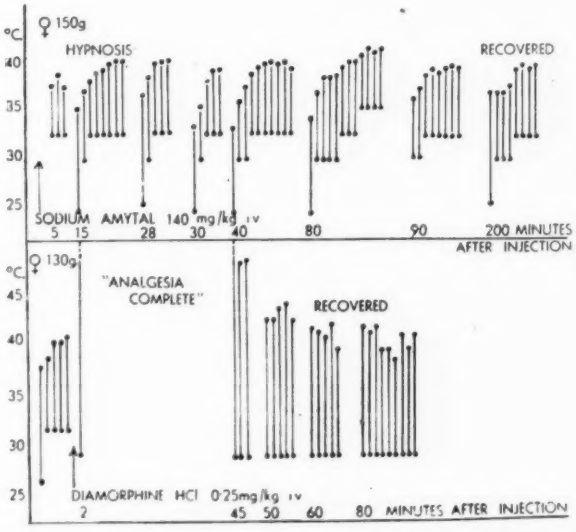

FIG. 3.—*Upper graph*. The temperature of reaction of the rat tail to thermal stimulation under hypnosis. Sodium amytal, 140 mg./kg., i.v. *Lower graph*. The temperature of reaction after intravenous diamorphine (0.25 mg./kg.) for comparison. Successive stimuli were applied at intervals of about one minute, commencing at the times indicated. Each vertical line represents the temperature range covered by the stimulus.

of the stimulus. By varying the heater current the same threshold is obtained irrespective of a long or short period of heating ; this holds also in man.

The effect on the reaction temperature of a number of analgesic drugs has been studied. After a suitable intravenous dose the " analgesic " effect develops so rapidly that readings after one minute almost invariably show the maximum effect ; the fall to normal levels is also quite a rapid process. With smaller doses threshold

increments varying from animal to animal are obtained. The rise in threshold may be very transient and appear only in tests carried out after one or five minutes. Davies, Raventos, and Walpole (1946) took readings fifteen minutes after intravenous injections. " Analgesic " effects of shorter duration, easily detectable by the present method, would not be recorded by their technique. Since their method is based upon measuring the reaction time to an uncontrolled stimulus, it is possible that the marked fall in tail temperature which occurs after such drugs may be responsible in part for prolonging the reaction time.

The dose-response curves for six drugs are shown in Fig. 4. A comparison of the potencies of these substances on the basis of " intensity " of effect has been made by estimating the dose required to produce the maximum response in 50 per cent of a group of animals (AD50) at any of the specified times of testing (Table II). Two successive elevations of the threshold to maximal values are stipulated in order

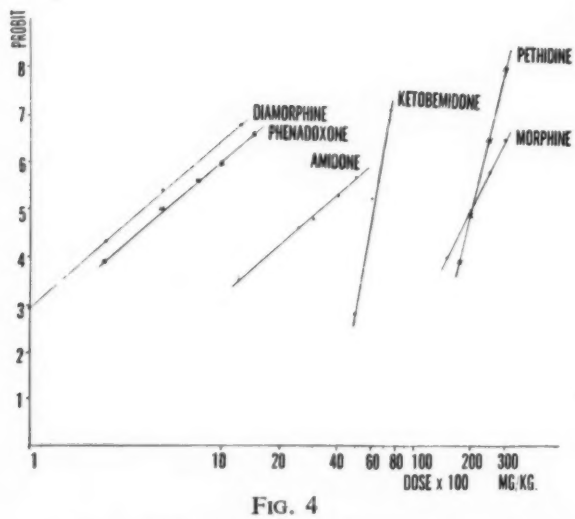

FIG. 4

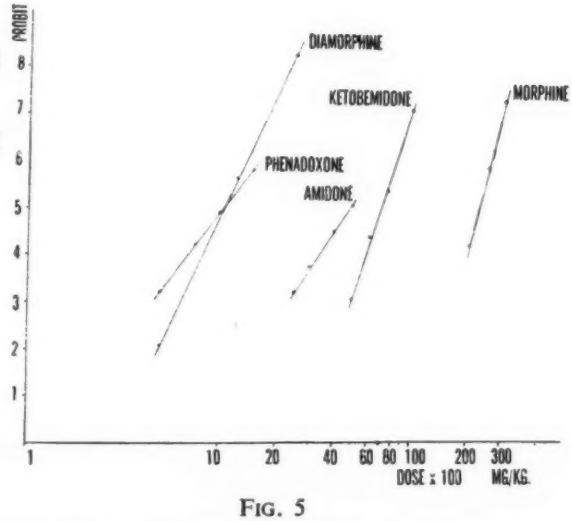

FIG. 5

FIG. 4.—The relationship between dose of drug and intensity of " analgesic " effect expressed as probit-response lines. Each point on the curves represents the frequency of occurrence of the maximal elevation of threshold as defined, in the 15 minutes following intravenous administration of the drug.

FIG. 5.—Probit-response lines for six analgesic drugs expressing the relation between dose and the number of animals showing a continued state of maximum " analgesia " for at least 15 minutes after intravenous administration of the drug.

to avoid occasional false readings which may occur. The " duration " of effect has also been assessed for the same dose range in terms of the amount of drug which will maintain the defined maximum level of " analgesia " for at least 15 min. in 50 per cent of a group of animals. The probit-response curves on this basis are presented in Fig. 5, and the AD50 values in Table III.

The intravenous toxicities of some of these drugs (Table IV) were determined in groups of five animals for each dose level (in a volume dose of 0.1 ml. per 100 g. weight). The mortality was recorded in deaths occurring within 24 hours.

Discussion

The intravenous analgesic potencies of morphine and pethidine according to Davies, Raventos, and Walpole (1946) are 2.2 and 4.6 mg./kg. respectively, as compared with 2.0 and 2.2. mg./kg. using the technique described in this paper. Since the above authors measured their analgesic effect 15 min. after injection and the present paper illustrates the brief duration of analgesic effect after intravenous pethidine (Table III), it is not surprising that Davies *et al.* found a higher dose of pethidine was required. Thorp (1946) and Basil, Edge, and Somers (1950), also using a radiant heat stimulus, administered their drugs subcutaneously so that rather larger doses of morphine were required to produce the required analgesic effect compared with the intravenous route (see below). From the present series of tests the interesting observation appears that diamorphine and phenadoxone are some fifty and forty times as potent respectively as morphine. These are much greater relative effects than previous workers have found, as the following figures show :

	Activity (morphine=1)			A.D.50 Morphine
	Phenadoxone	Diamorphine	Amidone	
Thorp (1946)	—	7	1.83	3.5
Basil *et al.* (1950)	3.8	—	1.3	3.67
Jackson	40	50	6.5	2.0

This enhancement of potency is clearly related to the route of administration, since the efficiency of morphine is not much altered by a change from subcutaneous to the intravenous route. It may be that phenadoxone and diamorphine are rapidly eliminated or destroyed so that the slower absorption from a subcutaneous site results in a relatively greater loss of potency compared with intravenous administration. The dose response curves for pethidine and ketobemidone are very steep and parallel, which is interesting in view of their similar chemical structure. A two-fold increase in dose is approximately adequate to cover the range of zero to 100 per cent " analgesia " for these two substances and morphine, whereas diamorphine and phenadoxone require about a tenfold increase. Amidone appears to fall in an intermediate range between these two groups. The ability of these drugs to maintain the maximum " analgesic " effect for at least 15 min. after injection is illustrated in

TABLE II

The intravenous potency of analgesic drugs in rats to a contact heat stimulus, based on the intensity of analgesic effect

Drug	AD50 (intensity) mg./kg.	Limits (P=0.05)	Activity ratio (morphine=1)
Morphine	2.0	1.7–2.4	1
Diamorphine	0.04	0.02–0.06	50
Phenadoxone	0.05	0.03–0.07	40
Pethidine	2.0	1.8–2.2	1
Ketobemidone	0.62	0.47–0.64	3.2
Amidone	0.31	0.20–0.80	6.5

TABLE III

The intravenous potency of analgesic drugs in rats to a contact heat stimulus, based on the " duration " of maximum analgesic effect

Drug	AD50 (" duration ") mg./kg.	Limits (P=0.05)	Activity ratio (morphine=1)
Morphine	2.2	1.9–2.8	1
Diamorphine	0.11	0.06–0.12	20
Phenadoxone	0.10	0.09–0.14	22
Pethidine*	—	—	—
Ketobemidone	0.69	0.59–0.82	3.2
Amidone	0.48	0.40–1.4	4.6

* In no single animal over the dose range investigated did the analgesic effect persist for 15 minutes.

Fig. 5 and Table III for the same dose ranges as in Fig. 4. Diamorphine and phenadoxone are still much more effective drugs in this sense than the others. The surprising failure of pethidine to produce a sustained response has already been mentioned ; otherwise the analgesics maintain the same order of potency as in Table II.

In view of the enhanced potency of diamorphine and phenadoxone by the intravenous route it seemed of interest to determine the relative toxicities of the analgesics under investigation by the same route. The results obtained (Table IV) illustrate the

TABLE IV

INTRAVENOUS TOXICITY OF ANALGESIC DRUGS

Drug	Dose mg./kg.	24 hr. deaths	% mortality
Morphine	40	0/5	0
	60	0/5	0
	80	0/5	0
	100	1/5	20
	120*	2/5	40
Diamorphine	5	0/5	0
	10	0/5	0
	20	2/5	40
	30	4/5	80
	35	5/5	100
	50	5/5	100
Phenadoxone	6.25	0/5	0
	12.5	2/5	40
	18.75	5/5	100
	25.0	5/5	100
Pethidine	12.5	0/5	0
	17.5	0/5	0
	20.0	1/5	20
	25.0	3/5	60
	40.0	5/5	100
Ketobemidone	2.5	0/5	0
	5.0	2/5	40
	12.5	3/5	60
	15.0	4/5	80
	25.0	5/5	100

* Limit of solubility at the intravenous dose volume of 0.1 ml./100 g. wt.

well-known tolerance of the rat for morphine, as it was impossible for solubility reasons to administer a dose greater than 120 mg./kg. and maintain the same dose volume as had been used throughout. The LD50 appeared to be about 140 mg./kg. The other drugs examined were much more toxic, their dose-response curves were steep and lay quite close together. The LD50 (mg./kg.) values were as follows : diamorphine, 22.5 ; pethidine, 22.5 ; phenadoxone, 12.5 ; and ketobemidone, 10. That diamorphine and phenadoxone do not show any relative enhancement of toxicity by the intravenous route suggests that the analgesic effects of drugs in the rat is not a manifestation of general toxicity. It would appear, moreover, that diamorphine and phenadoxone should be safer drugs to use intravenously for the relief of pain. It would be very interesting to have clinical observations on the relative efficacy of analgesics by intravenous administration.

The main object of this work was to devise some technique which would enable a comparison of the thermal responses of man and the rat to be made, and the comparative efficacy of analgesic drugs to be determined. It appears, however, that the method described in this paper possesses some advantages over techniques described by previous workers. The results obtained with a similar technique applied to man appear in a subsequent paper.

SUMMARY

1. A technique is described for evaluating the potency of analgesic drugs in the rat, using a thermal stimulus and measuring the temperature of reaction.

2. The normal temperature of reaction is about 40° C. Under the influence of an analgesic drug the temperature of reaction may rise up to a maximum of about 48° C. Beyond this temperature tissue damage occurs. Should no tail reaction have occurred when 48° C. is reached, it is unlikely to be elicited at higher temperatures. Attainment of this temperature is thus regarded as a maximum " analgesic " effect.

3. The intravenous potencies of various analgesic drugs are compared in terms of intensity and duration of effect. After intravenous injection of one of these drugs the maximum effect is reached almost invariably within one minute.

4. Whereas administration of morphine, pethidine, and amidone intravenously appears to have little effect on the analgesic potency, compared with the subcutaneous route, diamorphine and phenadoxone show greatly enhanced activity when injected intravenously, both as regards intensity and duration of effect.

5. The intravenous toxicities of morphine, diamorphine, phenadoxone, pethidine, and ketobemidone have been estimated. The enhanced analgesic potency of diamorphine and phenadoxone by the intravenous route is not due to any relative increase in toxicity compared with the other analgesics.

It is a pleasure to acknowledge indebtedness to Professor A. D. Macdonald, at whose suggestion this work was undertaken and for his continued interest in its progress.

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THE EFFECT OF ANALGESIC DRUGS ON THE SENSATION OF THERMAL PAIN IN MAN

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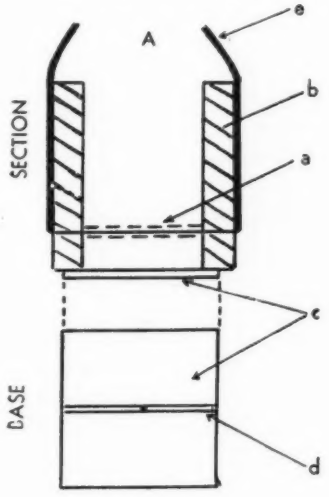
An analgesic drug would be expected to be capable of diminishing the perception of a suitable painful stimulus in the normal human subject. In past years, much effort has been expended in this direction using a variety of stimuli applied to the surface of the body—mechanical, electrical, and thermal. In all, the principle has been to apply to the subject an increasing measurable stimulus of relatively short duration until the sense of pain is recognizable. This stimulus is called the pain threshold. After the administration of a drug, the need to increase the intensity of the stimulus in order to elicit the same intensity of pain is considered to indicate an analgesic effect. The intensity and duration of such effects are studied at various dose levels.

An examination of previous publications reveals a natural tendency to base conclusions on too few subjects, sometimes on the authors only. The morphine group of analgesics and their synthetic substitutes are notorious for their side-effects which might influence the perception of a noxious stimulus so that an adequate number of subjects must be tested and the stimulus used must easily be recognized. No method so far evolved has withstood critical examination by other investigators. The failure of experimental methods may well be related to the application of stimuli of short duration to the body surface, whereas clinical pain is so often related to deep structures and is more sustained. In the absence of a satisfactory method of assessing analgesic potency in man, the relative efficacy of these compounds is usually related to morphine on the basis of animal tests of questionable validity.

The present paper is concerned with the effect of analgesics on the thermal pain sense in man. In theory, thermal stimuli possess the great advantage that the quantity or intensity of stimulus applied can be measured with accuracy. In practice, exact measurement of a thermal stimulus applied to the skin is difficult. Whilst the quantity of heat supplied to a given area of skin can be estimated with accuracy, a fraction of this, of unknown and varying magnitude, is dissipated by the blood supply to the area and in evaporating sweat, instead of being used to raise the temperature of the nerve endings. Thus, whilst control experiments carried out in surroundings of uniform temperature and humidity on a given skin site of constant initial temperature (e.g. the centre forehead) may give uniform threshold determinations (Hardy, Wolff, and Goodell, 1940a ; Schumacher, Goodell, Hardy, and Wolff, 1940), the marked autonomic effects of analgesic drugs (flushing, pallor, sweating) are liable to produce alterations in the thermal capacity of the skin of unknown and irregular magnitude (*vide infra*).

Hardy, Wolff, and Goodell (1940a) used an apparatus with which they and others have made extensive studies on the cutaneous sensations of heat and heat-pain (Oppel and Hardy, 1937 ; Hardy, Wolff, and Goodell, 1940, 1943a, 1943b, 1945 ; Schumacher, 1943 ; Wolff, 1947). For their studies on analgesic drugs these authors measured the quantity of heat required to produce a painful sensation on the centre of the forehead using a constant exposure to the stimulus of three seconds duration. After administration of a drug, threshold estimations were carried out at intervals of ten minutes and any increase in the quantity of heat required to elicit pain was regarded as an analgesic effect. This method suffers from the disadvantages referred to above. For example, Bigelow and Harrison (1944), studying the analgesic effect of intravenous procaine, noted that analgesia varied according to the subjective and objective disturbances produced by the drug. Whilst the results published by Hardy, Wolff, and Goodell (1940b) and others appeared to be very satisfactory, various workers have either recorded their inability to use the method (Dodds, Lawson, Simpson, and Williams, 1945 ; Thorp, 1946) or obtained results varying considerably from those of these authors (Christensen and Gross, 1948).

In the work here described, the skin contact temperature was measured during the application of a thermal stimulus. Temperature measurement is a more reliable method of estimating the intensity of a thermal stimulus and the results can be directly compared with those obtained in the rat by a similar technique (Jackson, 1952). Several modifications of the radiant heat technique have been applied to rats (Andrews and Workman, 1941 ; Smith and D'Amour, 1941 ; Ercoli and Lewis, 1945 ; Thorp, 1946), but the results are not directly comparable with those obtained in the human subject.



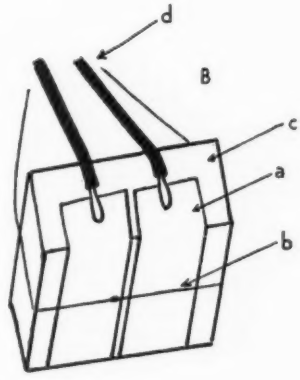


FIG. 1.—Apparatus for applying a known thermal stimulus to the skin. (A) *a*, heating element wound on a mica plate. *b*, asbestos mount. *c*, copper plate. *d*, thermocouple. *e*, heater and thermocouple leads. (B) *a*, heater element. *b*, thermocouple. *c*, mount. *d*, heater and thermocouple leads.

METHODS AND MATERIALS

Two forms of contact heater were used (A and B, Fig. 1). The first consists of a smooth, square copper plate (3 cm. square, 1 mm. thick) heated by radiation from a small electrically heated Nichrom element placed a few millimetres distant. The copper plate ensures that the heating of the skin in contact with it is uniform. Interposed between the copper plate and skin is a fine copper-constantan thermocouple connected to a sensitive recording galvano-

meter. This stimulus involves a reaction time of about 25 to 30 seconds using a current of 2 ampères at 10 volts provided by a transformer from the mains supply. A few minutes are required for cooling so that single threshold estimations can be performed at intervals of about five minutes.

The second type of heater used (B, Fig. 1) consists of two strips of uniform Nichrom resistance tape (0.006 inch thick) connected in series and arranged side by side so as to form a heating surface of 2.5 sq. cm. For use it is strapped to the skin and an alternating current of 5 to 6 amperes at 10 volts is required to produce a suitable rate of heating. The skin contact temperature is again measured by a thermocouple of low thermal capacity running transversely across the heater. The thermal capacity of the heater is minimal, for immediately the current is discontinued the temperature ceases to rise and cooling is rapid. Stimuli may therefore be applied in rapid sequence when necessary.

It is obviously most important to ensure uniformity of heating and an accurate response of the thermocouple. The transverse position of the latter was chosen to minimize heat loss along the wires.

The 23 subjects used in these experiments were of both sexes, mainly of the student age group; a few were of middle age. They were recumbent during the experimental period. Control determinations were first made until the temperature of pain perception was constantly recognized. The subject expressed vocal appreciation of the end-point and an observer noted the galvanometer reading. Various sites of the body were chosen for different experiments, but for prolonged tests the lateral aspect of the calf was commonly used. In earlier experiments isolated threshold estimations were made at intervals of about ten minutes, as was the practice of previous workers using radiant heat (Hardy, Wolff and Goodell, 1940a), but later more frequent stimuli were applied.

Drugs were given subcutaneously, intramuscularly, or intravenously; some subjects took part in only one experiment, whilst others submitted to several tests at varying dose levels or had different drugs. Nitrous oxide inhalations were made from a Douglas bag containing gas mixtures of known composition, analysed by the excellent method described by Chaney and Lombard (1932). The gas mixture was administered through a nasal inhaler.

There are various local and general factors which might influence the pain threshold level in any subject. The more important are psychological effects, the rate and frequency with which the stimulus is applied, the site of stimulation, and the initial skin temperature. These are referred to later.

Results

In estimating the pain threshold it is of importance that the element of pain should be recognized as soon as possible—yet it has to be sufficiently sharp to be easily perceptible in spite of any peculiar sensations which might follow administration of a drug. The sensations produced by the heating elements used correspond closely with those described by Wolff and his collaborators (1940a)—a sense of warmth, replaced by increasing hotness and followed by a distinct throb of pain which is recorded as the threshold. Should heating be continued further, the sense of pain increases with surprising rapidity, and soon becomes intolerable to most subjects. The thermal threshold for pain has been investigated by other workers. Weber (1846) considered that the temperature required to produce pain was so high that, acting for a little time upon nerve, it limited conduction; he gave this temperature as 48.7° C. Lewis and Love (1926) included a study of thermal pain in their investigations of cutaneous sensations and reactions. They found the element of pain normally commenced at a surface temperature of 43° C. or a subcutaneous temperature of

38° C. and that these temperatures are close to those producing an injury flare. They maintained that 43° C. was intolerable to most skins under conditions of asphyxia which allowed the heat to penetrate, or 42° to 43° C. if the thermocouple was inserted into the skin. More recently, Lloyd-Smith and Mendelssohn (1948) determined the skin temperature exposed to radiant heat for 15 minutes which produced a pricking pain and recorded a temperature of 45° C. By means of the heater A (Fig. 1) the mean thresholds for a number of subjects of both sexes were calculated from single stimuli delivered at intervals of about ten minutes ; the results are presented in Table I. Some of these may also be seen in graphical form in Fig. 2. It was found

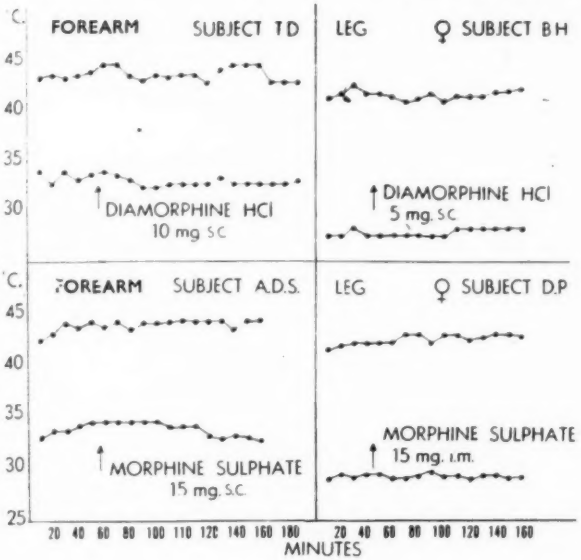


FIG. 2

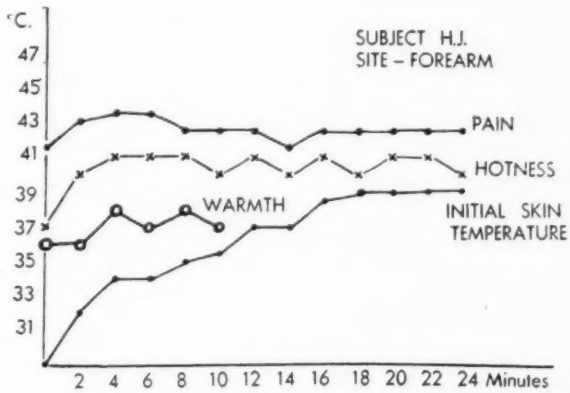


FIG. 3

FIG. 2.—Four examples of the failure of subcutaneous morphine and diamorphine to affect thermal pain perception. Single estimations of the threshold (the upper series of points in each graph) were made at intervals of about 10 min. from the initial skin temperatures indicated (lower points in each case). The first few points establish uniformity of the normal response before administration of the drug at the times indicated. No significant change in pain threshold occurred subsequently in these subjects or in any of a total of 15 individuals tested.

FIG. 3.—The effect of changes of initial skin temperature on the perception of thermal sensations. The temperature of the forearm was steadily increased by means of an electrically heated pad, and estimates of the sensations of warmth, hotness and pain made at intervals of 2 min. Although the initial skin temperature rose during the experiment by 10° there was no effect on the perception of thermal pain or the sensation of hotness. The onset of warmth was perceptible until the skin temperature reached the warmth threshold at about 37°.

that the initial temperature of the skin has little or no influence on the pain threshold with occasional stimuli. In the experiment summarized in Fig. 3, the temperature of the arm was slowly raised by means of an electrically heated pad whilst repeated estimates of the threshold were made ; the onset of the sensations of warmth and hotness was also noted. When the arm temperature reached 36° C. the sense of warmth was no longer perceptible, whereas the perception of hotness and pain remained at steady levels throughout. When stimuli are applied in quite rapid succession, e.g. one per minute, for considerable periods of time, the threshold tends

to be somewhat higher (compare Tables I and II). These periods of stimulation do not produce any noticeable damage beyond an erythema ; Lewis (1942) commented that the onset of pain occurred at a temperature at which tissue damage commenced

TABLE I

NORMAL THERMAL THRESHOLDS

Single stimuli at intervals of 10 min.

Subject	Site	Temperature °C.		Number of stimuli
		Initial	Mean threshold	
D.P. (f.)	Leg	30	41.5	8
E.B. (f.)	Leg	29	42.25	9
K.H. (f.)	Leg	29	41.25	14
M.B. (f.)	Leg	29	41.0	5
M.B. (f.)	Leg	29	43.0	5
J.A. (f.)	Leg	29	40.0	10
J.H. (m.)	Forehead	35	44.0	6
H.J. (m.)	Forehead	34	42.5	8
T.D. (m.)	Forehead	34	43.5	4
T.D. (m.)	Forehead	34	44.5	5
L.K. (m.)	Forehead	35	43.5	4
L.K. (m.)	Forehead	35	43.5	2
A.G. (m.)	Forehead	35	44.5	6
A.S. (m.)	Forehead	35	44.0	5
H.J. (m.)	Forehead	35	43.0	9
H.J. (m.)	Forehead	35	43.5	10
F.H. (m.)	Leg	30	41.25	6

TABLE II

NORMAL THERMAL THRESHOLD

Site: Arm and leg. Initial temp. 34.5. Stimulation: 1 per min.

Subject	No. of stimuli	Mean threshold (°C.)
H.J. (m.)	29	45.0
H.J. (m.)	19	45.0
H.J. (m.)	6	44.0
H.J. (m.)	12	46.5
H.J. (m.)	10	45.5
L.K. (m.)	19	45.0
A.H. (f.)	45	42.0
A.H. (f.)	27	43.5
L.K. (m.)	12	46.0
L.K. (m.)	4	45.5
S.D. (f.)	19	44.0
S.D. (f.)	7	44.0
S.D. (f.)	10	42.0
E.H. (f.)	14	43.5
J.L. (m.)	9	44.5
D.D. (m.)	10	43.0
G.W. (m.)	15	45.5
J.G. (f.)	20	45.0
F.H. (m.)	7	44.0
F.H. (m.)	4	45.5
A.P. (m.)	8	45.0

as manifest by flare production. In some subjects the threshold varied from day to day as much as 2° C., but remained constant during an experimental period. If the skin is damaged with an erythema dose of ultra-violet light, the threshold soon falls, even down to blood temperature, and recovers in the course of a few days (Fig. 4).

It became apparent that no significant change occurred in the thermal pain threshold in most subjects after therapeutic doses of analgesic drugs (Fig. 2) when single stimuli were applied at intervals of ten to fifteen minutes. The possibility that the stimulus used was too intense appeared unlikely in view of the observation that individuals recognizing a pain threshold two or three degrees lower than most were also unaffected by these doses of analgesic drugs and also that the sense of " hotness," when recorded, also remained unimpaired.

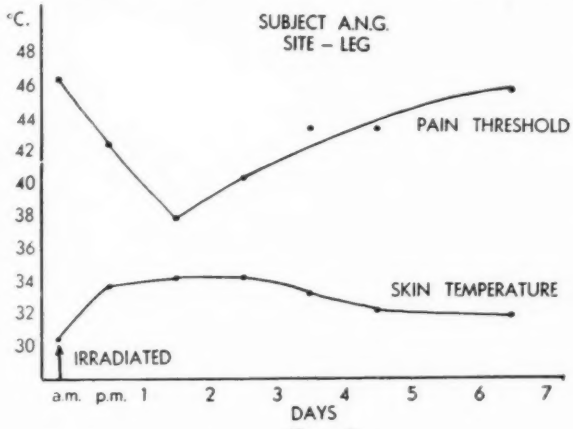


FIG. 4

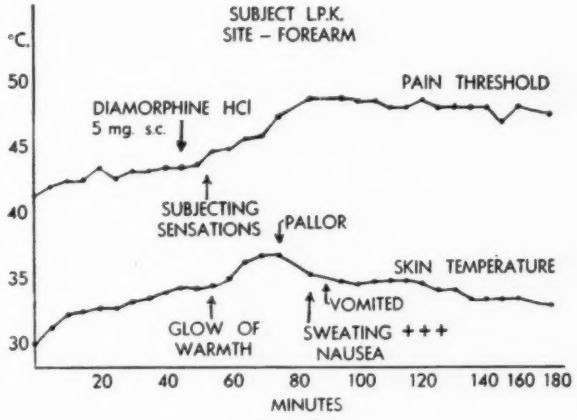


FIG. 5

FIG. 4.—This experiment illustrates the depression of thermal pain threshold (upper curve) almost down to blood temperature, which takes place after a small area of skin is given an erythema dose of ultra-violet light. A few days is required for restoration to normal levels. Note the associated rise in skin temperature due to the erythema.

FIG. 5.—An unusual response to the subcutaneous injection of diamorphine. This subject showed elevation of the thermal threshold (upper curve) associated with a severe vasomotor-type of reaction. The persistent elevation of the threshold is apparently due to damage to the skin which occurs at this temperature (48°).

Only one subject (L.P.K.) showed a progressive increase in threshold and that occurred after the administration of diamorphine for the first time (Fig. 5). Soon after the drug had been given, he showed a striking vasomotor reaction—intense pallor, severe sweating—and vomited once. The threshold rose to 48° C. and persisted at this level. After 140 minutes an adjacent site was heated and gave normal threshold readings, so that obviously the repeated stimulation to 48° C. had impaired the sensibility of the skin. In a repetition of this experiment a few weeks later, a similar but less intense reaction occurred, but there was no alteration in threshold.

In further attempts to demonstrate an effect of analgesics on pain threshold, stimuli were applied in rapid succession to see whether analgesic drugs produce some kind of fatigue phenomenon. In one series of experiments the initial heating to threshold was followed by three or four others in quick succession from a constant initial temperature and the mean value calculated. Intervals of about ten minutes

were allowed between each series of heatings (Fig. 6). Finally, stimuli were applied steadily at intervals of about one per minute from a constant initial temperature for prolonged periods of time (Fig. 7). In both these groups of experiments, morphine and diamorphine were administered intravenously. Three of the seven subjects who

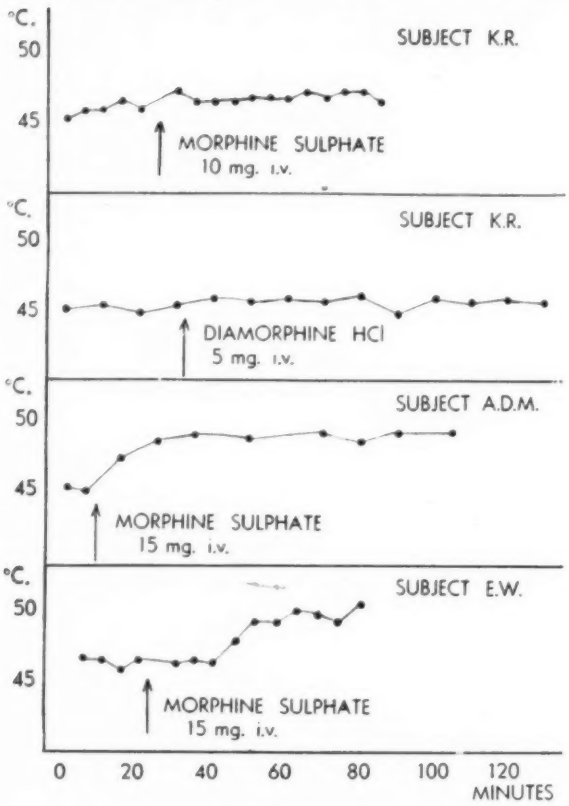


FIG. 6

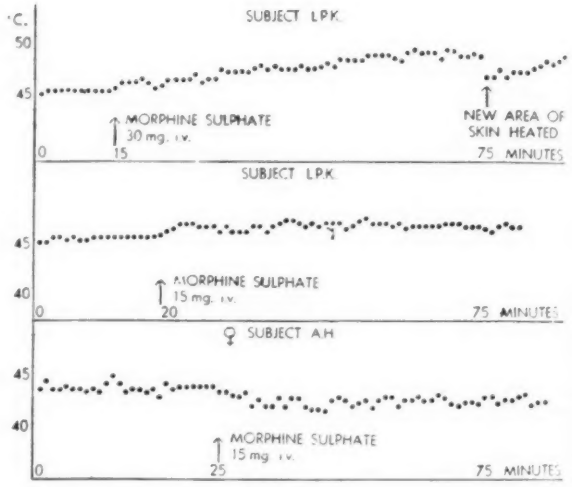


FIG. 7

FIG. 6.—The intravenous injection of morphine and diamorphine and the perception of thermal pain. Four examples are shown in which each point on the graphs represents the mean of at least three estimates of the thermal threshold made in rapid succession from an initial skin temperature of 35°. Marked elevation of threshold occurred in subjects A.D.M. and E.W. Subject K.R. maintained his normal threshold level throughout.

FIG. 7.—The perception of thermal pain after intravenous morphine. Estimates of the thermal threshold were made at intervals of 1 min. Subject L.P.K. showed a noticeable rise after a dose of 15 mg., whilst 30 mg. elevated the threshold to a level considered intolerable by normal individuals. On the other hand the threshold of A.H. (lowest graph) became lower after 15 mg. of the drug.

submitted to the experiments did show a progressive rise in threshold to relatively large doses of morphine. The maximum temperature reached was in the region 48° to 49° C. The duration of effect is unfortunately unknown, since successive heating to these levels damages the skin ; thus, stimulation of a fresh area of skin after an hour or so in each case gave normal readings.

The side-effects which accompanied the use of these drugs, especially the large intravenous doses, made further experiments on this scale unjustified.

The temperature of maximum tolerable thermal pain

Using a copper bar as heat reservoir, Lewis and Love (1926), with two subjects, recorded a surface temperature of 47° to 48° C. as intolerable for more than 5 seconds. These authors stated that if the skin temperature is raised gradually, a surface temperature of 51° to 52° C. may be borne, but related this to coincident damage impairing the sensation. In the present experiments, the heater was strapped in position, and, starting from a constant initial temperature of 37° C., estimations of the pain threshold were made, each being followed by continued heating until the subject found the pain intolerable. In the subjects tested (four in number), the maximum achieved was 48° to 49° C. Even after more than ten such heatings, the perception of pain in the heated area remained normal, although the area was obviously damaged and subsequently developed a blister (Fig. 8). The duration of each heating was about 15 seconds.

The effect of asphyxia on the perception of thermal pain

Using the radiant heat technique, Hardy, Wolff, and Goodell (1940a) maintained that occlusion of the circulation in the arm was followed by a progressive increase in the pain threshold of the hand to a maximum of about 30 per cent at the end of the 35-minute period of asphyxia. When the circulation was restored, the threshold rapidly returned to normal. With the same technique, Thorp (1946) found a peak of " analgesia " occurred after the pressure was released (50 per cent increase), the rise during the asphyxial period being about 30 per cent. Lewis (1942), however, had previously stated that production of a state of asphyxia in a limb lowered the perception of thermal pain on the skin of the limb, because arresting the circulation allowed heat to penetrate more easily ; a skin temperature of 42° to 43° C. was said to be intolerable under these circumstances. According to Lewis, therefore, a state of hyperalgesia rather than analgesia is produced. Fig. 9 illustrates the effect of asphyxia using thermal stimuli applied to the forearm in rapid sequence from a constant initial temperature. The threshold diminishes until a fairly steady value is reached about 2° lower than the control readings. After restoration of the circulation, there is a rapid and transient increase in threshold which corresponds to the period of severe " pins and needles " which naturally occurs at this time and makes the perception of the thermal stimulus difficult. On the basis of this typical experiment, together with Lewis's observation, it seems that the radiant heat technique in the studies referred to above can give misleading results.

The inhalation of nitrous oxide and thermal pain

The inhalation of even relatively small percentages (e.g. 20 to 30 per cent) of nitrous oxide produces well-marked subjective sensations and objective changes in the human subject. Marked analgesic effects are stated to occur with moderate percentages (45 to 50 per cent) in obstetrical cases. Seevers, Bennett, Pohle, and Reinardy (1937) studied the effect of nitrous oxide on the pain threshold to pin-prick as measured by the Frey hair technique. In the present experiments, it was expected that the thermal pain threshold would show progressive elevation until the subject became uncooperative but not unconscious. However, the threshold proved remarkably resistant to change, even in a number of subjects who had no previous

experience of inhaling volatile anaesthetics. About 70 per cent of nitrous oxide was required to cause any change and the maximum threshold observed prior to unconsciousness was again 48° to 49° C. (Fig. 10).

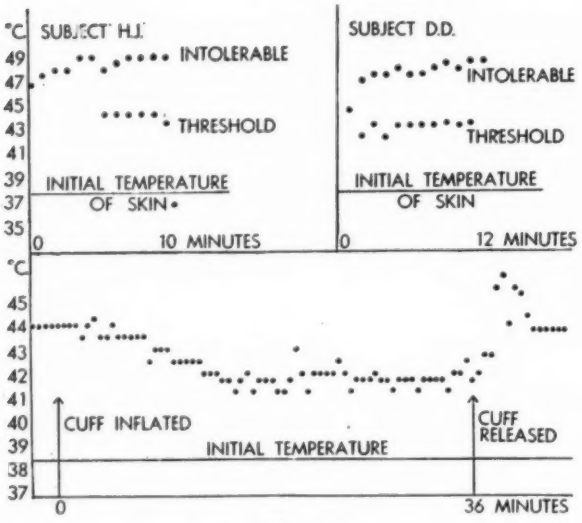


FIG. 9

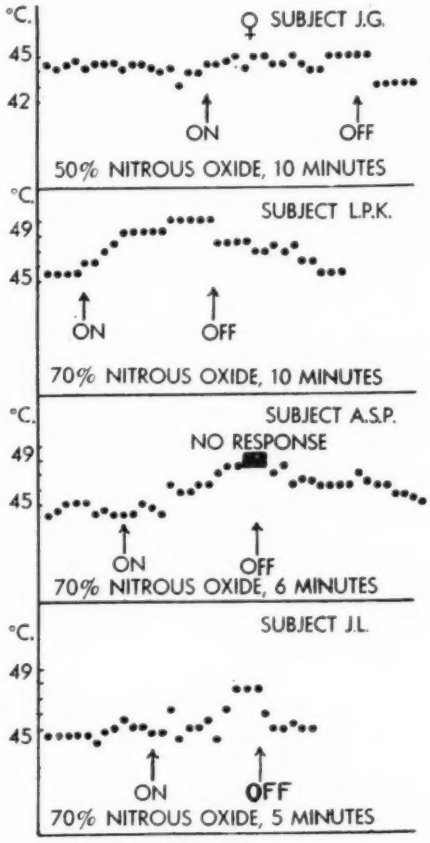


FIG. 10

FIG. 8.—The maximum tolerance for thermal pain. Successive heatings of the skin were made to the level of intolerability (upper points) from an initial temperature of 38°. Although the skin was obviously damaged at the conclusion of the experiments (about 10 min. in each instance) and subsequently blistered, the perception of thermal pain remained unimpaired (lower points).

FIG. 9.—The effect of asphyxia on the perception of thermal pain. The thermal threshold was estimated on the forearm at half-min. intervals, and the circulation occluded by means of a cuff. Note the progressive fall in pain threshold (over 2°) which followed. When the circulation was restored after 36 min. the threshold rises temporarily but soon returns to normal.

FIG. 10—Examples of the effect of nitrous oxide on thermal pain perception. The points represent the pain thresholds obtained by successive skin heatings from an initial skin temperature of 38°. In general, 70 per cent of the gas was required to elevate the threshold significantly without producing unconsciousness. The maximum threshold registered by any subject (10 tested) was about 49°.

DISCUSSION

The results presented above show that the temperature at which a thermal stimulus is first perceived by the human subject is unaffected by therapeutic doses of morphine or diamorphine. In addition, it has proved surprisingly difficult to elevate this threshold temperature either by the induction of asphyxia in the area

stimulated or by inhalation of nitrous oxide. Intravenous injection of larger doses of morphine or diamorphine has produced rather ill-defined elevation of the thermal threshold by 3 or 4° C. in three of seven subjects tested ; damage to the skin soon occurs at these higher temperatures. It appears that the maximum rise in temperature which may occur is to the 48°–49° C. level. This limitation is probably imposed by the factors of maximal stimulation of nerve endings and concurrent damage. As a result of this relatively small temperature rise, the large dose of opiate required, the uncertainty of the response in different subjects, and the unpleasant side-effects of the drugs (vomiting especially), the use of thermal stimulation for the assessment of the analgesic potency of drugs is impracticable.

The results of some previous workers using radiant heat as the stimulus, in which smooth and well-defined " analgesic " effects were obtained with therapeutic doses of morphine and related compounds, must have some other interpretation. It is established that widely varying results have been obtained by different groups of workers using such a technique. How then may these findings be reconciled with the readiness with which thermal pain perception is apparently abolished in the rat, as judged by the tail reaction ?

In man, thermal pain is perceived in the region of 43° C., using occasional stimuli ; in the rat under similar circumstances, the tail reaction occurs at about 38° to 40° C. (Jackson, 1952). This latter zone of temperature corresponds roughly with the sense of hotness in man, which the animal is likely to interpret as noxious. Doses of analgesic drugs, not far removed from those used in man on a weight basis, readily elevate the reaction temperature in rats to 48° C. A tail response above this level is rarely obtained and skin damage will occur ; " analgesia " is thus complete. This temperature is similar to the maximum threshold (48° to 49° C.) observed in human subjects treated with analgesics or nitrous oxide and to the temperature at which a thermal stimulus becomes intolerable. It therefore seems reasonable to suppose that thermal analgesic tests in rats do in fact measure " analgesia." The lack of a similar effect on threshold in man could be a question of magnitude of dose.

Summary

1. Temperature measurement is claimed to be the most satisfactory method of determining the intensity of a thermal stimulus applied to the skin.

2. On this basis, the threshold to thermal pain in man and the tail response in the rat are similar.

3. The thermal threshold in man is very resistant to elevation. In most subjects tested, therapeutic doses of analgesic drugs had no significant effect. Asphyxia for 40 minutes in a limb lowers the threshold temperature.

4. Relatively large intravenous doses of morphine and diamorphine raised the threshold by as much as four degrees centigrade (to 48° to 49° C.) in a minority of subjects. This increase appears to be the maximum attainable in man, and corresponds with the maximum temperature of response observed in the rat (48° C.).

5. The magnitude of the dose required to elevate the threshold, the small change which may occur, and the uncertainty of the response in different subjects imply that in man thermal stimulation of the skin is an unsuitable method for assessing the analgesic potency of drugs.

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THE EFFECT OF SOME OXIDATION PRODUCTS OF PHENOTHIAZINE ON LIVER FLUKE (*FASCIOLA HEPATICA*) *IN VITRO*

BY

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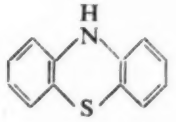
Phenothiazine (I) is used extensively as an anthelmintic and has proved to be very efficient in the removal of some intestinal worms in sheep. A considerable amount of research has been carried out in order to ascertain the fate of phenothiazine when administered to ruminants (Collier, 1940 ; Swales and Collier, 1940 ; Lipson, 1940 ; Collier, Allen, and Swales, 1942 ; Collier, Allen, and Swales, 1943 ; Clare, 1947 ; Harpur, Swales, and Dunsteht, 1950).

The dose of phenothiazine required appeared to be unnecessarily large when its insolubility was considered, and Taylor and Sanderson (1940) studied the possibility of the formation in the host of another substance of greater anthelmintic potency than phenothiazine.



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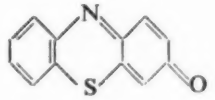

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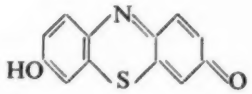

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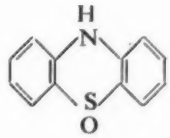

(IV)

A number of oxidation products have been found in the host dosed with phenothiazine, viz., phenothiazone (II), thionol (III), or their easily oxidizable leuco-compounds, phenothiazine sulphoxide (IV), and certain conjugates, e.g., leuco-phenothiazone conjugated with sulphuric acid.

Phenothiazone was administered to sheep by Collier, Allen, and Swales (1943), but found to have no effect on nematodes removable by phenothiazine, and both phenothiazone and thionol were tested by Taylor and Sanderson (1940) in goats heavily infected with parasitic worms, but no anthelmintic effect was observed.

As no mention was made of the liver fluke (*Fasciola hepatica*) in these experiments, the investigation described in the present paper was carried out to ascertain the action, if any, of phenothiazone, thionol, and phenothiazine sulphoxide on liver fluke *in vitro*.

Methods

Preparative.—Phenothiazone, thionol, and phenothiazine sulphoxide were prepared by the methods of Olivier and Combé (1950), Houston, Kester, and DeEds (1949), and Barnett and Smiles (1909) respectively.

Biological testing.—The liver fluke was tested *in vitro* by the kymographic technique of Baldwin (1943) as modified by Chance and Mansour (1949). By frequent changes of the Ringer solution (1.5–2 hr.), it was possible to keep the liver fluke in a lively condition for 7 to 8 hr., after removal from the host. Aeration of the Ringer solution had no effect on prolonging the life of the parasite.

The tests were carried out at 37–38° C. with concentrations of 1 : 1,000 and less till the phenothiazine oxidation products ceased to have an effect on the helminth. As the compounds under test were very insoluble in water, emulsification by Baldwin's method was attempted. Phenothiazine sulphoxide formed only a suspension at the concentrations used.

Results

It was found that phenothiazone had a lethal effect from a concentration of 1 : 1,000 to 1 : 8,000 and a paralysant effect from 1 : 8,000 to 1 : 16,000, whilst thionol and phenothiazine sulphoxide had no lethal action, and were paralysant at 1 : 1,000 and from 1 : 1,000 to 1 : 4,000 respectively.

Figs. 1–4 show the kymographic records of the three compounds at the minimum concentration required to produce a lethal or paralysant effect. The time is marked in minutes on the signal line, and the movement of the helminth in the Ringer solution is shown from the beginning of the experiment to the first long stroke, when the Ringer solution was replaced by the emulsion or suspension of the compound. At the second long stroke on the signal line, amphetamine sulphate (1 : 5,000) in Ringer's solution, as recommended by Chance and Mansour (1949), replaced the compound. Restoration of the movement of the parasite with amphetamine sulphate indicates that the compound had a paralysant effect, whilst no movement shows that the substance was lethal.

The results are summarized in Table I.

TABLE I

THE LOWEST CONCENTRATIONS AT WHICH OXIDATION PRODUCTS OF PHENOTHIAZINE EXERT A LETHAL OR PARALYSANT EFFECT ON THE LIVER FLUKE *in vitro*

Compound	Nature of preparation	Concentration	Effect
Phenothiazone	Emulsion	1 : 8,000	Lethal
	,,	1 : 16,000	Paralysant
Thionol	,,	1 : 1,000	,,
Phenothiazine sulphoxide	Suspension	1 : 4,000	,,

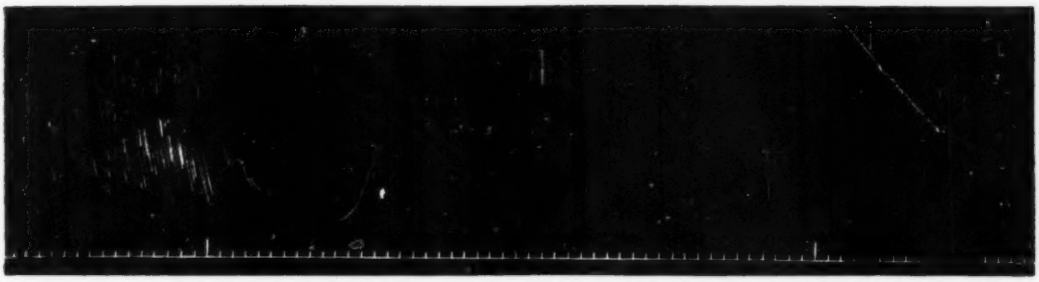

FIG. 1.—Lethal effect of phenothiazone (1 : 8,000). No response to amphetamine sulphate.

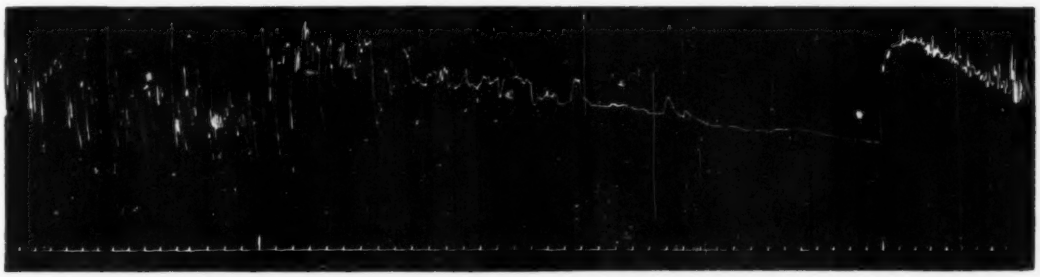

FIG. 2.—Paralysant effect of phenothiazone (1 : 16,000) after 25 min., followed by response to amphetamine sulphate.

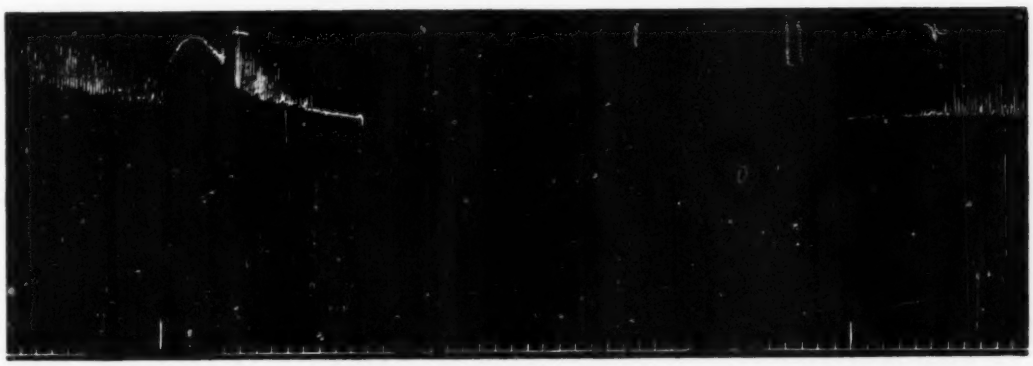

FIG. 3.—Paralysant effect of thionol (1 : 1,000), followed by response to amphetamine sulphate.

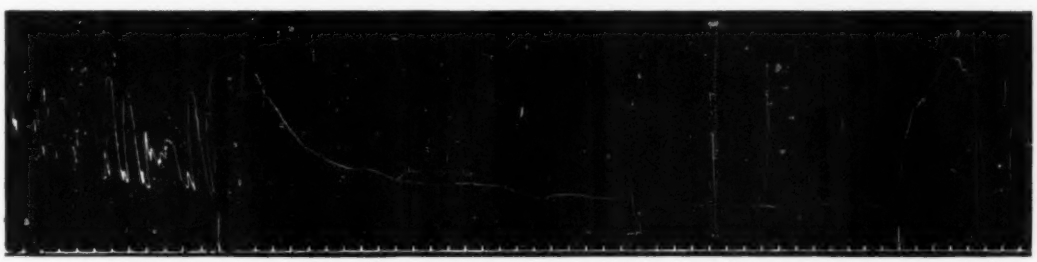

FIG. 4.—Paralysant effect of phenothiazine sulphoxide (1 : 4,000), followed by response to amphetamine sulphate.

Discussion

The outstanding feature of this investigation is the discovery of the lethal effect of the phenothiazone at 1 : 8,000 and its paralysant effect at 1 : 16,000. Chance and Mansour (1949) also found that certain substances, which were paralysants at lower concentrations, produced lethal effects at higher concentrations.

As a paralysant phenothiazone is sixteen times as potent as thionol and four times as effective as the sulphoxide. It should be pointed out, however, that the potency of phenothiazine sulphoxide is not strictly comparable with the other two compounds, since an effective concentration was only achieved by using a suspension.

It would appear, therefore, that the introduction of a hydroxyl group in position 7 of the phenothiazone molecule reduces its paralysant effect to one-sixteenth of its value and destroys its lethal effect.

It is significant that phenothiazine has no paralysant or lethal effect *in vitro* (Chance and Mansour, 1949), and therefore additional experiments *in vivo* with phenothiazine and its oxidation products would be desirable.

Summary

1. Phenothiazone, thionol, and phenothiazine sulphoxide, oxidation products of phenothiazine, have been prepared and tested at various concentrations against liver fluke (*Fasciola hepatica*) *in vitro*.

2. Phenothiazone had a lethal effect at a concentration of 1 : 8,000 and was paralysant at 1 : 16,000. Thionol and phenothiazine sulphoxide had only paralysant effects at 1 : 1,000 and 1 : 4,000 respectively.

We are indebted to the Agricultural Research Council for a grant which defrayed the cost of this investigation and to Mr. Soulsby, of the Edinburgh City Abattoir, for the collection of parasites. Our thanks are also due to Dr. Parnell for his interest in this investigation.

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THE INFLUENCE OF GROUPS IN THE MOLECULE OF 2 : 3-DIHYDRO-3-KETOBENZO-1 : 4-THIAZINE ON ITS EFFECT ON LIVER FLUKE (*FASCIOLA HEPATICA*) *IN VITRO*

BY

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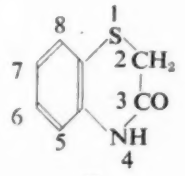
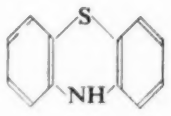
(Received January 17, 1952)

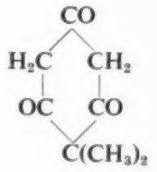
The parent compound chosen for the investigation described in this paper was 2 : 3-dihydro-3-ketobenzo-1 : 4-thiazine (I), as it contains in its molecule certain features present in substances which possess anthelmintic properties, e.g. phenothiazine (II). It also contains a $-CH_2CO-$ unit, which is a characteristic feature in filicic acid (III), the most important of the organic acids present in the anthelmintic *Filix mas*.



(I)



(II)



(III)

On account of these similarities, it was reasonable to assume that 2 : 3-dihydro-3-ketobenzo-1 : 4-thiazine might show some anthelmintic activity, and consequently this compound and a number of its derivatives with substituents in the 6- and 6 : 7-positions were tested against liver fluke (*Fasciola hepatica*) *in vitro*, so that the influence of the various groups on anthelmintic activity could be compared.

METHODS

Preparative.—2 : 3-Dihydro-3-ketobenzo-1 : 4-thiazine and derivatives were prepared by methods described elsewhere (Mackie and Raeburn, 1952). Unfortunately it was not possible to prepare the 6-bromo-derivative analytically pure, so that a complete comparison of the influence of the four halogens could not be made.

Biological testing.—The method of *in vitro* testing adopted was Baldwin's kymographic technique (1943) as modified by Chance and Mansour (1949) for liver fluke.

The maximum concentration used was 1 : 1,000, either as solution, emulsion, or suspension, and the tests were carried out at lower concentrations till no effect on the helminth was observed. The minimum effective concentration was noted for each compound, so that it was possible to arrange most of the substituent groups in order according to their effect on potency. There were, however, a few compounds whose minimum effective concentration could not be determined owing to their insolubility, for even at 1 : 4,000 heavy suspensions were obtained.

At least four determinations were carried out on every compound at each concentration, unless the compound had no effect, when two tests were considered sufficient. Moreover, two or sometimes three determinations were carried out at the same concentration for a given compound with different batches of parasites. Except in a very few determinations, the kymographic records for a given compound at a particular concentration were remarkably similar in form.

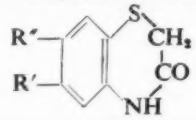
Results and Discussion

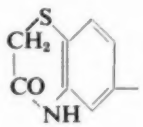
2 : 3-Dihydro-3-ketobenzo-1 : 4-thiazine and its derivatives, which have been fully tested *in vitro*, have shown a paralysant effect on liver fluke. The most potent was the 6-chloro-derivative, which produced a paralysant effect at 1 : 8,000, the minimum effective concentration. At a concentration of 1 : 1,000 the 6-amino- and 6-mercapto-compounds had so powerful an effect that the addition of amphetamine produced only small irregular movements after 10 min. At 1 : 2,000, the 6-amino-derivative produced a paralysant effect for about 20 min., which was then followed by small convulsive movements.

The 6-arsonic and -stibonic acids, the 6-chloromercuri-derivative, and bis-(2 : 3-dihydro-3-ketobenzo-1 : 4-thiazin-6-yl) were very insoluble and the minimum

TABLE I

The minimum effective concentration is the lowest at which a paralysant effect was observed.
Derivatives of 2 : 3-dihydro-3-ketobenzo-1 : 4-thiazine



Compound	R′	R″	Nature of preparation	Minimum effective concentration
2 : 3-Dihydro-3-ketobenzo-1 : 4-thiazine	H	H	Emulsion	1 : 2,000
6-Amino- ,, ,, ...	NH_2	H	,,	1 : 1,000
6-Amino- ,, ,, HCl	NH_2,HCl	H	Solution	1 : 3,000
6-Acetoamino- ,, ,, ...	$CH_3CO.NH$	H	Suspension	1 : 1,000
6-Fluoro- ,, ,, ...	F	H	,,	1 : 2,000
6-Chloro- ,, ,, ...	Cl	H	Emulsion	1 : 8,000
6-Iodo- ,, ,, ...	I	H	Suspension	1 : 5,000
6-Thiocyano- ,, ,, ...	CNS	H	,,	1 : 1,000
6-Triazo- ,, ,, ...	N_3	H	Emulsion	1 : 6,000
6-Nitro- ,, ,, ...	NO_2	H	,,	1 : 4,000
6-Nitroso- ,, ,, ...	NO	H	,,	1 : 6,000
6-Mercapto- ,, ,, ...	SH	H	,,	1 : 1,000
6-Arsonic Acid ,, ,, ...	H_2AsO_3	H	Suspension	Not obtained
6-Stibonic Acid ,, ,, ...	H_2SbO_3	H	,,	,, ,,
6-Chloromercuri- ,, ,, ...	HgCl	H	,,	,, ,,
6 : 7-Dimethoxy- ,, ,, ...	CH_3O	CH_3O	Emulsion	1 : 4,000
6 : 7-Dihydroxy- ,, ,, ...	OH	OH	,,	1 : 1,000
Bis-(2 : 3-dihydro-3-ketobenzo-1 : 4-thiazin-6-yl)		H	Suspension	Not obtained

effective concentration was not obtained. At a concentration of 1 : 4,000, the 6-chloromercuri-compound had a powerful depressant action but was not completely paralysant, the 6-arsonic acid showed a slight depressant effect, but the 6-stibonic acid and bis-(2 : 3-dihydro-3-ketobenzo-1 : 4-thiazin-6-yl) had no effect.

The results are summarized in Table I.

A typical kymographic record is shown in Fig. 1. This illustrates the effect of the compound on the helminth. The normal rhythmic movement of the worm in Ringer's solution at 37–38° C. is shown from the beginning of the experiment to the first long stroke on the signal line marked in minutes, when an emulsion of the compound under test replaced the original Ringer's solution. Amphetamine sulphate (1 : 5,000) in Ringer's solution, as employed by Chance and Mansour (1949), replaced the compound after approximately 45 to 50 min., as indicated by the second long stroke on the signal line. Restoration of the movement of the parasite on addition of amphetamine sulphate indicated that the compound was paralysant.

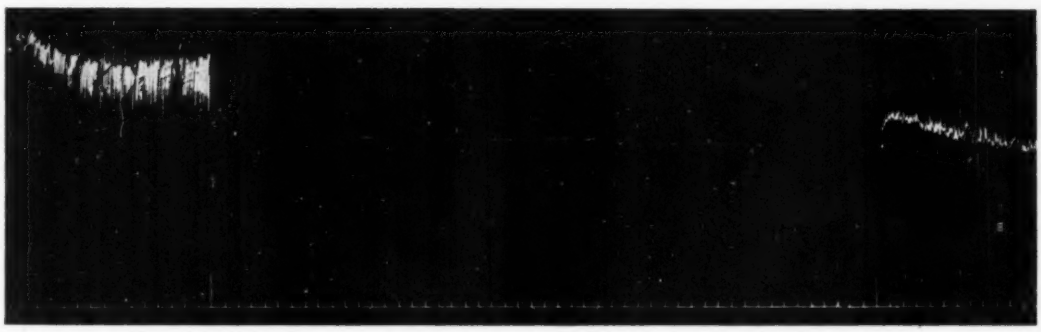


FIG. 1.—Paralysant effect of 6-chloro-2: 3-dihydro-3-ketobenzo-1 : 4-thiazine (1 : 8,000), followed by response to amphetamine sulphate (1 : 5,000).

In the following remarks it should be pointed out that comparisons between solutions, emulsions, and suspensions are not strictly accurate, but may be considered to be approximate (cf. Baldwin, 1943).

The introduction of the amino-group lowers the anthelmintic potency of 2 : 3-dihydro-3-ketobenzo-1 : 4-thiazine by one half, but the amino-hydrochloride is three times more effective than the free base ; this difference may, however, be due to free acid in solution. Acetylation does not appreciably alter the potency of the base. This is perhaps surprising, as acetylation might be expected to effect some change.

With the exception of fluorine, which does not affect the activity of the unsubstituted compound, the introduction of halogen atoms (Cl or I) increases its potency. The chloro-derivative is four times as effective as the unsubstituted compound, the order of potency of the halogens being Cl>I >F.

The triazo- and nitroso-groups also have a considerable influence on anthelmintic activity. It is particularly interesting to note that the latter group is 1.5 times as effective as the nitro-group.

The thiocyano- and mercapto-groups both halve the activity of the unsubstituted compound.

The anthelmintic potency of the unsubstituted compound is increased twofold by the introduction of two methoxy-groups in the 6 : 7-positions. The corresponding dihydroxy-derivative, however, is only half as effective as the unsubstituted compound ; this was unexpected, as many examples are known where the introduction of a hydroxy-group increases the activity. It is possible that the vicinal position of the hydroxy-groups may be a controlling factor.

The very insoluble derivatives, the 6-arsonic and 6-stibonic acids, the 6-chloromercuri-compound, and bis-(2 : 3-dihydro-3-ketobenzo-1 : 4-thiazin-6-yl) might have had a paralysant effect at concentrations higher than 1 : 4,000 if they had been more soluble.

From the results obtained, it will be seen that the order of potency of the radicals is as follows : Cl>N_3,NO >I >NO_2, 6 : 7-dimethoxy >NH_2,HCl > unsubstituted, F>NH_2, CH_3.CO.NH, CNS, SH, 6 : 7-dihydroxy.

The results of testing 2 : 3-dihydro-3-ketobenzo-1 : 4-thiazine and its derivatives suggest that the constitutional similarities of this compound to phenothiazine and to filicic acid, already noted, have some significance. Although Chance and Mansour (1949) found phenothiazine to be without effect on liver fluke *in vitro*, they did find that *Filix mas* was lethal at a concentration of 1 : 5,000.

Summary

1. 2 : 3-Dihydro-3-ketobenzo-1 : 4-thiazine and derivatives with substituents in the 6- and 6 : 7-positions were tested against liver fluke (*Fasciola hepatica*) *in vitro* and the effects recorded kymographically.

2. All the compounds, which could be tested over a wide range of concentrations, showed paralysant effects.

3. Minimum effective concentrations (MEC) were estimated and it was possible to arrange the substituent groups in the following order of potency (MEC in parentheses) : Cl(1 : 8,000)>N_3, NO(1 : 6,000) >I(1 : 5,000) >NO_2, 6 : 7-dimethoxy (1 : 4,000)>NH_2HCl(1 : 3,000)>unsubstituted compound, F(1 : 2,000)>NH_2, CH_3.CO.NH, CNS, SH, 6 : 7-dihydroxy (1 : 1,000).

4. The 6-amino- and 6-mercapto-derivatives were paralysant at a concentration of 1 : 1,000, and severely reduced the subsequent response to amphetamine sulphate.

5. Owing to the insolubility of the 6-arsonic acid, 6-stibonic acid, 6-chloromercuri-derivatives and bis-(2 : 3-dihydro-3-ketobenzo-1 : 4-thiazin-6-yl), no minimum effective concentration could be obtained, but a 1 : 4,000 suspension of the 6-chloromercuri-derivative produced almost complete paralysis ; the 6-arsonic acid was slightly depressant and the other two derivatives without effect at this concentration.

It is a pleasure to acknowledge our indebtedness to the Principal and Governors of the Heriot-Watt College for providing the laboratory facilities for this investigation and to the Agricultural Research Council for financial aid. Our thanks are also due to Professor Bell for his interest in this work, to Mr. A. W. Patterson for his helpful advice in the biological testing, and to Mr. Soulsby, of the Edinburgh City Abattoir, for the collection of the parasites.

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THE MECHANISM OF ANTICURARE ACTION OF CERTAIN NEOSTIGMINE ANALOGUES

BY

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From the Department of Pharmacology, Middlesex Hospital Medical School, London, W.1

(Received November 27, 1951)

Recently Randall and Lehmann (1950) and Randall (1950) reported on a number of neostigmine analogues which had a striking anticurare action in cats and dogs *in vivo* but were only weak cholinesterase inhibitors *in vitro*. Their findings were confirmed by McFarlane, Pelikan, and Unna (1950) and Artusio, Riker, and Wescoe (1950) in man, and by Riker and Wescoe (1950) and Wescoe, Riker, and Brothers (1949) in cats. The compounds these authors had studied were either hydroxy anilinium salts or esters of these salts (excluding the carbamic esters). It was found that these substances differed from neostigmine in that the peak of anticurare action was reached within a minute after the injection and that side effects, e.g. salivation and bradycardia, were either negligible or absent. A further advantage was that they could be given at relatively short intervals without loss of their anticurare potency or a decrease in the sensitivity of the endplate to *d*-tubocurarine.

It is now generally held that tubocurarine blocks neuromuscular transmission by competing with acetylcholine for the receptors on which the latter acts. In this way it raises the threshold for stimulation. This blocking effect of tubocurarine can be overcome by anticholinesterases, dyes, potassium salts and phenols, or by substances which stimulate the endplate, each type of compound exerting an anticurare action by a different mechanism. There was difficulty in attributing the action of the neostigmine analogues to any of the known anticurare mechanisms. Randall and Lehmann (1950) thought that the anticholinesterase activity—about 1/100th or less of that of neostigmine—was too small to account for the anticurare action, which was about one quarter that of neostigmine ; these authors also excluded a stimulant action on the endplate, because, although some of the compounds caused a contracture of the muscle in small concentrations, others had no stimulant properties whatsoever. Riker *et al.* (1949), Wescoe *et al.* (1949), Artusio *et al.* (1950), and McFarlane *et al.* (1950), studying only compounds with good stimulating properties, took the view that the anticurare action could be entirely accounted for by a stimulant action on the endplate. Randall (1950) in continuation of his work came to similar conclusions, but also considered the possibility that the free phenolic group, present in many of the compounds, might be of importance.

In the experiments to be described I have studied four of the neostigmine analogues in normal and curarized cats, on the isolated phrenic nerve diaphragm of the rat, on the rat intestine, and on the frog rectus.

Methods and Materials

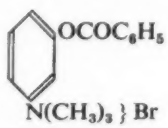
Cats were anaesthetized with 80 mg. chloralose/kg. (i.v.) or 50 mg. pentobarbitone/kg. (i.p.). Records of the tension of the tibialis anterior muscle were taken on a smoked drum by means of an isometric steel spring myograph. The muscle was stimulated by supramaximal square pulses of 0.25 msec. duration, applied to the nerve by shielded silver electrodes. Injections were given i.v. by means of a cannula inserted into the superficial jugular vein.

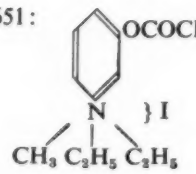
The phrenic nerve diaphragm preparation of the rat (Bülbring, 1946) was set up in Tyrode solution containing 0.2 per cent (w/v) glucose and aerated with a 95 per cent $O_2 + 5$ per cent CO_2 mixture.

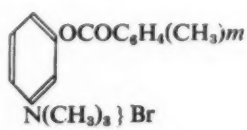
The frog rectus was set up as described by Chang and Gaddum (1933). The isolated rat intestine was suspended in aerated Tyrode solution and the contractions of the longitudinal muscle alone were recorded.

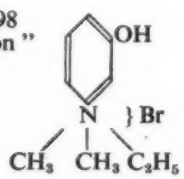
For estimating anticholinesterase potency *in vitro* the manometric technique (Ammon, 1933) was used. Lysed human red cells and human serum were used as enzyme sources and *dl*-acetyl-β-methylcholine (final concentration 0.03 M) and benzoylcholine (final concentration 0.01 M) as substrates. Red cell esterase is referred to in the text as cholinesterase I and serum cholinesterase as cholinesterase II.

The following neostigmine analogues were studied :

Ro 2–2650:  = 3-benzoxy-N-trimethyl-anilinium bromide.

Ro 2–2651:  = 3-acetoxy-N-methyl-diethyl-anilinium iodide.

Ro 2–2783:  = 3-m-toluyloxy-N-trimethyl-anilinium bromide.

Ro 2–3198 " Tensilon "  = 3-hydroxy-N-dimethyl-ethyl-anilinium bromide.

All four compounds were pure crystalline substances, readily soluble in water ; solutions of them were freshly made immediately before use. Neostigmine was used as the methylsulphate. Throughout the text the doses and concentrations given refer to the salts.

RESULTS

The actions of neostigmine analogues in cats

Curarized cats.—In cats in which the intravenous (i.v.) injection of *d*-tubocurarine had reduced the response of the muscle to indirect stimulation by 50 per cent or more the i.v. injection of Ro 2–2650, Ro 2–2651, Ro 2–2783, or Ro 2–3198 caused a rapid increase in twitch tension after a latent period of 30 to 60 sec. (Fig. 1). The anticurare activity of Ro 2–2651 and Ro 2–3198 was about a fifth of that of neostigmine ; Ro 2–2650 and Ro 2–2783 were slightly weaker, their potency being about a tenth to a fifteenth of that of neostigmine. With all four compounds a maximum anticurare action was reached within one to two minutes of injection, after which the twitch tension began to fall off ; this decay was more rapid with Ro 2–2651 and Ro 2–3198 than with the other two analogues. On the other hand, neostigmine differed from its analogues in that the maximum anticurare action with a given dose was not reached until six to ten minutes after the injection ; the twitch tension then remained constant and no recurrence of curarization was noticeable.

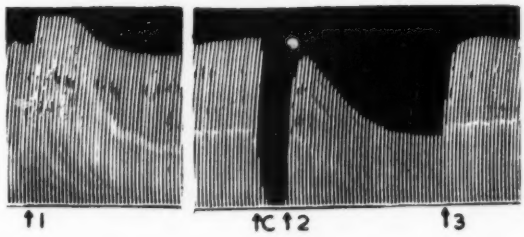


FIG. 1.—Cat, 3.7 kg. Pentobarbitone. Record from Musc. tibialis anterior. Frequency of stimulation 1 in 10 sec. ↑ : 1 mg. Ro 2–3198 i.v. at 1, 2, and 3. ↑ C: 700 μg. *d*-tubocurarine chloride i.v.

If decamethonium was used in order to interrupt neuromuscular transmission, neostigmine and its analogues did not restore twitch tension. These experiments exclude the possibility that the neostigmine analogues improve the twitch tension of a curarized muscle by an action beyond the endplate, i.e., by an action on the muscle fibre itself.

In order to see how much of the anticurare action was due to inhibition of cholinesterase cats were first given an injection of 2 mg. atropine per kg. and then 2–5 mg. tetraethylpyrophosphate per kg. were injected i.v. Such treatment completely abolished the anticurare action of neostigmine and its analogues. Acetylcholine, decamethonium, and choline, however, which are supposed to counteract the blocking action of tubocurarine by their stimulant action on the endplate (Wilson and Wright, 1937 ; Hutter, 1951) still restored the twitch tension to a considerable extent (Fig. 2).

Normal cats.—All the evidence obtained from the experiments described above suggested that the anticurare action of the neostigmine analogues was more likely to be due to cholinesterase inhibition than to an acetylcholine-like action upon the endplate. If that were so animals untreated with tubocurarine would be expected to show definite signs of cholinesterase inhibition after treatment with these drugs ; this was, however, only partly true.

In normal cats the i.v. injection of Ro 2–2651 and Ro 2–3198, in doses which had a significant anticurare action, caused a rapid increase in twitch tension up to 20 per cent above the original tension with a return to normal within three to five

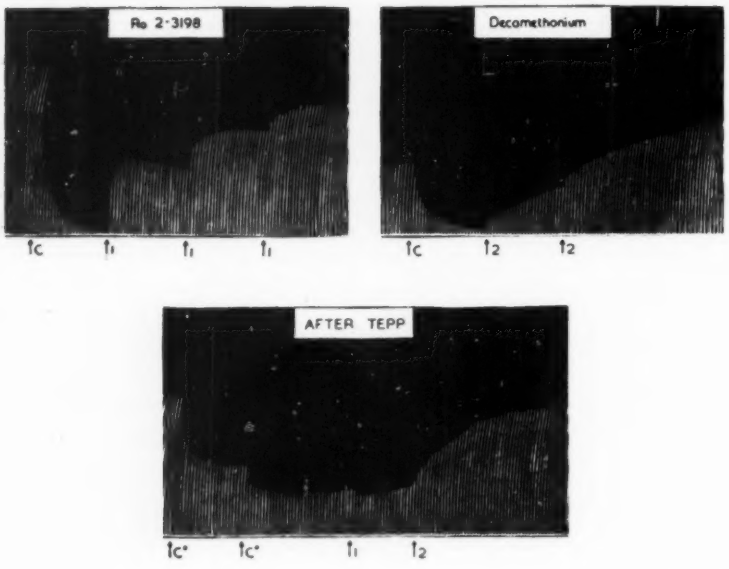


FIG. 2.—Cat, 3.5 kg. Chloralose. Record from Musc. tibialis anterior. Frequency of stimulation 1 in 10 sec.; 2 mg. atropine/kg. ↑C: 700 μg. *d*-tubocurarine chloride i.v. ↑C': 1 mg. *d*-tubocurarine chloride i.v. ↑1: 1 mg. Ro 2–2651 i.v. ↑2: 40 μg. decamethonium iodide i.v. TEPP: 5 mg. tetraethylpyrophosphate i.v.

minutes (Fig. 1). At the same time the cats showed fibrillations all over the body and action potentials taken from the muscle were markedly repetitive in character, but only a very slight increase in the activity of the viscera innervated by the parasympathetic system was noticeable. Ro 2–2650 and Ro 2–2783 increased the twitch tension to a much smaller extent, and likewise the muscle action potentials showed less marked changes than were seen with the two other analogues. Neostigmine, on the other hand, in doses which had the same anticurare action potentiated the twitch tension to a much greater extent (up to 50 per cent of the original tension), the maximum effect developing after a considerable time-lag. The muscle action potentials were of repetitive character and a marked increase in the activity of the viscera innervated by the parasympathetic system was seen.

In view of these results it was therefore desirable to obtain more information about the mechanism of anticurare action of the neostigmine analogues on the two preparations widely used for such a purpose, i.e., the isolated phrenic nerve diaphragm of the rat (Blaschko, Bülbring, and Chou, 1949) and the isolated frog rectus (Hobbiger, 1950).

The action of neostigmine analogues on the isolated phrenic nerve diaphragm of the rat

The neostigmine analogues could be divided into two groups according to their action on the normal diaphragm :

(*a*) The first group consisted of Ro 2–2651 and Ro 2–3198. Both these substances potentiated the twitch tension in doses of 1 μg./ml. and higher (Fig. 3). Compared with neostigmine the two analogues were about fifty times weaker in this property.

(*b*) The second group consisted of Ro 2–2650 and Ro 2–2783. These substances did not potentiate the twitch tension in any concentration. If the concentration

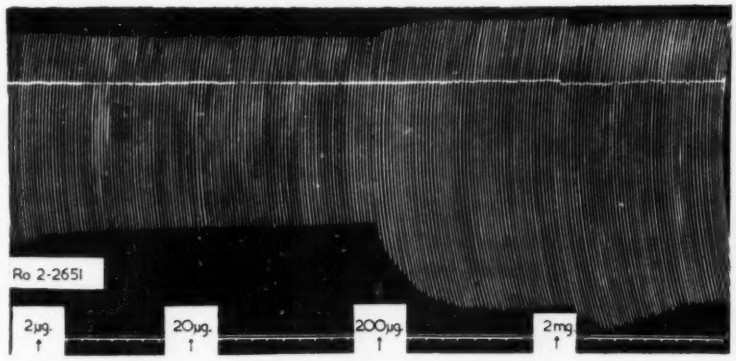


FIG. 3.—Isolated rat phrenic nerve diaphragm. Effect of Ro 2–2651 on the response to supramaximal stimulation of the nerve at a frequency of 1 in 10 sec. The figures give the total amount of Ro 2–2651 added to the bath (volume 100 ml.).

was as high as 10 µg./ml. the twitch tension of the diaphragm rapidly declined (Fig. 4). This effect was readily reversed by removal of the compounds from the bath fluid.

When the response of the diaphragm to tetanic stimulation was used as an indicator of cholinesterase inhibition (Burgen and Hobbiger, 1951) some recovery of the diaphragm was seen in the presence of Ro 2–3198 and Ro 2–2651 ; this was due to the disappearance of the compounds from the bath fluid, but the extent of this recovery was too small to account for the short duration of action *in vivo*.

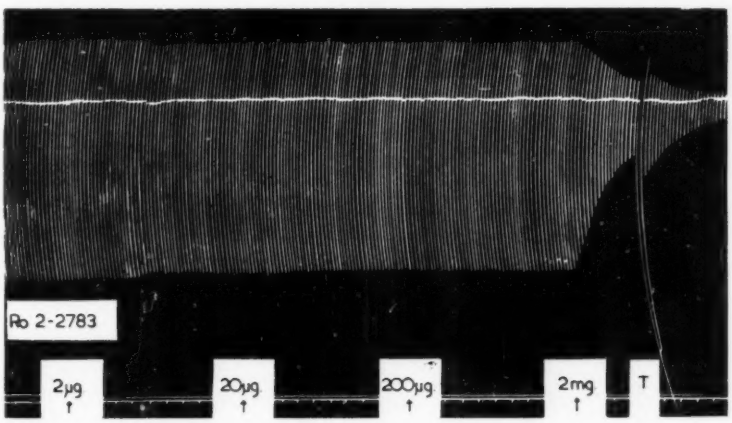


FIG. 4.—Isolated rat phrenic nerve diaphragm. Effect of Ro 2–2783 on the response to supramaximal stimulation of the nerve at a frequency of 1 in 10 sec. The figures give the total amount of Ro 2–2783 added to the bath (volume 100 ml.). T = Tetanic stimulation (100 stimuli/sec.) for 5 sec.

Anticurare action on the diaphragm

All four neostigmine analogues antagonized the actions of tubocurarine on the diaphragm in concentrations of 1 µg./ml. and higher. Ro 2–2650 and Ro 2–2783 were significantly weaker in this respect than Ro 2–2651 and Ro 2–3198. Again, as in the cat the onset of anticurare action was much more rapid with the neostigmine analogues than with neostigmine. With Ro 2–2650 and Ro 2–2783, once tubocurarine had reduced the twitch tension by 50 per cent or more, recovery was only partial, regardless of the dose used (Fig. 5). With Ro 2–2651 and Ro 2–3198 recovery was more complete and these drugs had about one-fiftieth of the anticurare potency of neostigmine (Fig. 6).

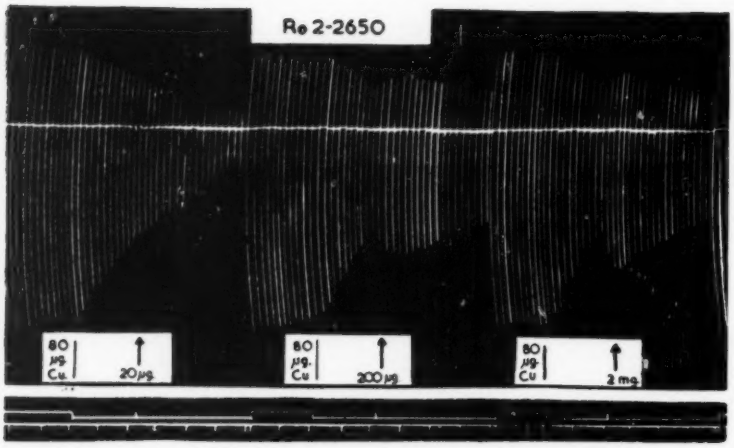
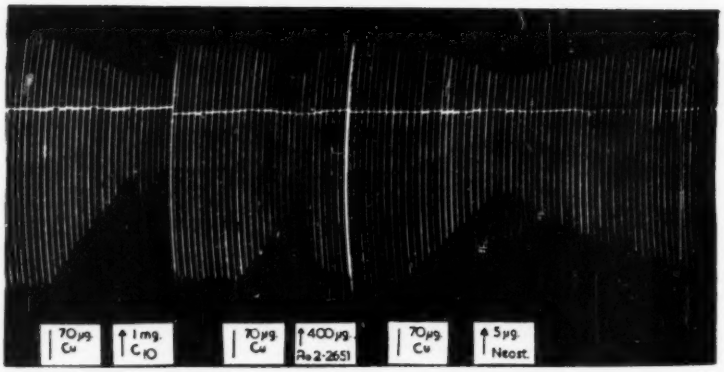


FIG. 5.—Anticurare action of Ro 2–2650 on the isolated phrenic nerve diaphragm preparation. 80 µg. Cu = 80 µg. *d*-tubocurarine chloride. 20 µg., 200 µg., and 2 mg. are the amounts of Ro 2–2650 added to the bath (volume 100 ml.). Time in min.

FIG. 6.—Anticurare action of decamethonium iodide (C_{10}), Ro 2–2651, and neostigmine methylsulphate on the isolated rat phrenic nerve diaphragm. The doses refer to total amounts added to the bath (volume 100 ml.).



For Ro 2–2651 and Ro 2–3198 the ratio of the stimulant to the anticurare dose was exactly the same as for neostigmine. The anticurare action of Ro 2–2650 and Ro 2–2783, however, could only be explained by assuming that these substances had two actions, one on cholinesterase and one on the endplate itself. On the normal diaphragm these actions would oppose each other, but in the presence of tubocurarine the anticholinesterase action might predominate because of the less favourable conditions for an action on the endplate. Such dual effects of Ro 2–2650 and Ro 2–2783 could be clearly demonstrated on the frog rectus.

To find out how much of the anticurare action of the neostigmine analogues was due to a stimulant action on the endplate, substances which act predominantly in such a way were studied. It was found that acetylcholine and choline had no anticurare action whatsoever and decamethonium gave only a very short-lasting improvement (Fig. 6), which was always much less than that seen with the neostigmine analogues.

Anticurare action on the diaphragm after DFP

In order to demonstrate that the anticurare action of the neostigmine analogues on the diaphragm was really due only to an inhibition of cholinesterase, the cholinesterase of the diaphragm was inactivated by di*iso*propylfluorophosphonate (DFP).

In these experiments DFP was added to the bath for one hour in a concentration of 20 μg./ml. A diaphragm which had been treated in this way still responded after removal of the DFP to single stimuli, but was unable to sustain a tetanus (Evans, 1951 ; Burgen and Hobbiger, 1951). One hour after removal of DFP and repeated washing no cholinesterase activity could be detected.

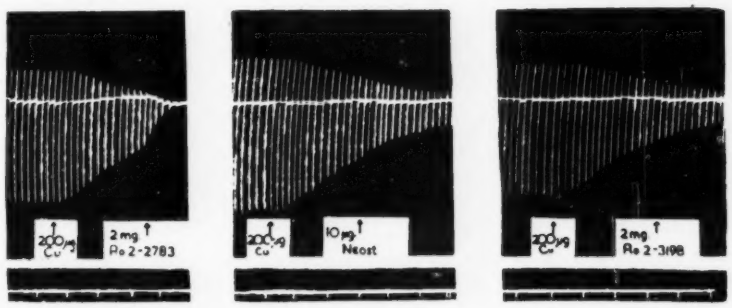


FIG. 7.—The action of Ro 2–2783, neostigmine methylsulphate, and Ro 2–3198 on the isolated phrenic nerve diaphragm after DFP (20 μg./ml. for 1 hr.). The doses refer to total amounts added to the bath (volume 100 ml.). Time in min.

As can be seen from Fig. 7, Ro 2–3198 and neostigmine had no longer any anticurare action on a diaphragm after inhibition of its cholinesterase by DFP ; the same was true with Ro 2–2651. Ro 2–2650, Ro 2–2783 (Fig. 7), and decamethonium potentiated the blocking action of tubocurarine. Catechol (Fig. 8) and KCl, on the other hand, were as effective anticurare agents as on a normal diaphragm. These experiments excluded the possibility that the neostigmine analogues act by virtue of their free phenolic groups (present in the original compounds or after enzymic hydrolysis of the esters) or by combination with tubocurarine itself.

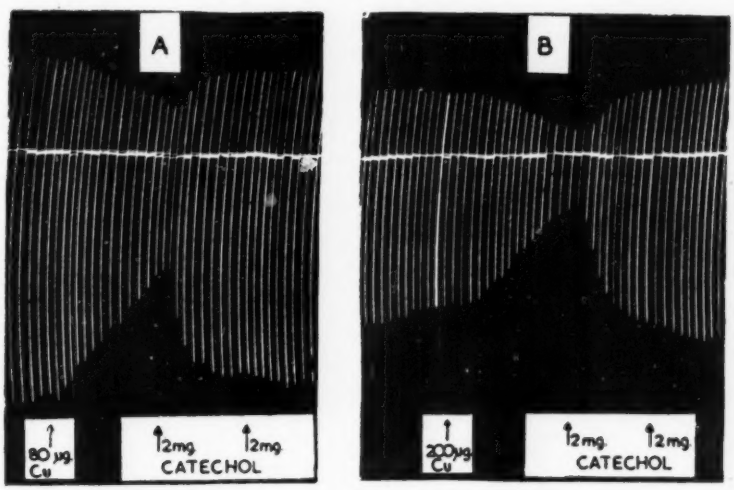


FIG. 8.—The anticurare action of catechol on the isolated phrenic nerve diaphragm, (A) before, and (B) after, treatment with DFP (20 μg./ml. for 1 hr.).

Action on the frog rectus

On the normal frog rectus abdominis the neostigmine analogues had practically no effect on the tonus in a concentration up to 100 μg./ml. Ro 2–2651 and Ro 2–3198 potentiated the actions of acetylcholine, the optimal concentration for this action being about 10 μg./ml. (Table I) ; with higher doses this sensitization was

again partly lost. By this test Ro 2–3198 had about a tenth of the potency of neostigmine, and Ro 2–2651 slightly less. Ro 2–2650 and Ro 2–2783 sensitized the rectus to acetylcholine to a much smaller degree, and in concentrations of 10 μg./ml. and higher they even depressed the response to acetylcholine (Table I). The effect of the neostigmine analogues (sensitization or depression) was rapid in onset and

TABLE I

Sensitization of the frog rectus to acetylcholine by neostigmine and its analogues. The changes in sensitivity are expressed in per cent of the original response to 1 μg. Ach/ml. (= 100). + = sensitization. − = depression

Drug	Change in sensitivity produced by 10^{-7}	10^{-6}	10^{-5}	10^{-4}
Ro 2–2650	—	+70	+100	< −90
Ro 2–2651	—	+150	+600	+700
Ro 2–2783	—	+25	−50	< −90
Ro 2–3198	—	+230	+660	+400
Neostigmine	+200	+750	+900	—

a maximum effect was achieved within one minute. With neostigmine, on the other hand, the sensitization of the rectus to acetylcholine was slightly greater than with Ro 2–2651 or Ro 2–3198 (Table I), but the rectus had to be in contact with a given solution for at least thirty minutes in order to show a maximum effect.

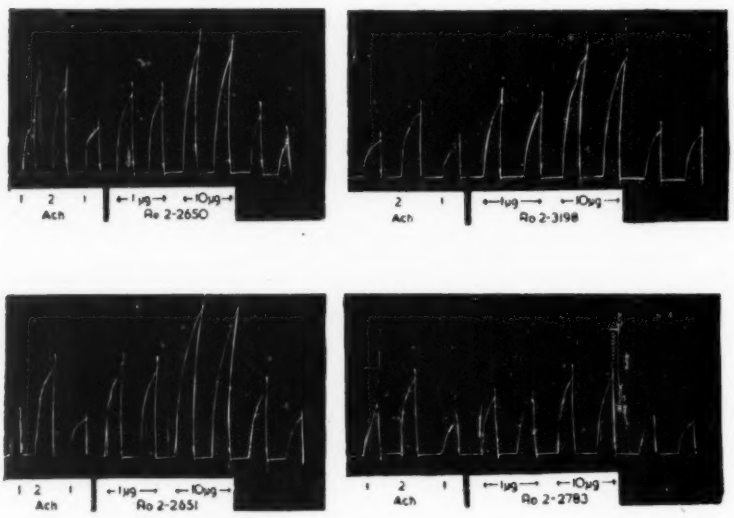


FIG. 9.—Anticurare action of the neostigmine analogues on the frog rectus. The doses give the final concentration. Duration of tests 60 sec. Interval between the tests 3 min. In the tests marked 1, 1 μg. acetylcholine/ml., and in the tests marked 2, 2 μg. acetylcholine/ml., were used to induce contracture. In the other tests the dose of acetylcholine was always 1 μg./ml. The concentration of *d*-tubocurarine chloride was 1 μg./ml. throughout the experiment.

On the curarized rectus muscle all four neostigmine analogues had a marked anticurare action (Fig. 9), which was already significant at dose levels of 1 μg./ml. As on the diaphragm this anticurare action was completely absent after inhibition of cholinesterase by tetraethylpyrophosphate (for method see Hobbiger, 1950); indeed the actions of tubocurarine were then even intensified by the substances (Fig. 10).

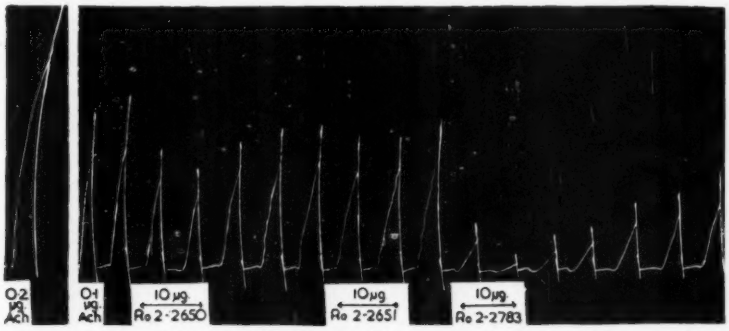


FIG. 10.—Anticurare action of the neostigmine analogues on the frog rectus previously treated with tetraethylpyrophosphate. Duration of tests 60 sec. Interval between the tests 3 min. In the first test 0.2 µg. acetylcholine/ml. was used, and in the subsequent tests 0.1 µg. acetylcholine/ml. Throughout the experiment 1 µg. *d*-tubocurarine chloride/ml. was present in the bath.

Substances with an acetylcholine-like action, on the other hand, e.g. choline or decamethonium, still produced a contracture of the rectus after inhibition of cholinesterase, and if acetylcholine was given during such a contracture a further shortening of the muscle occurred.

The anticurare action of the neostigmine analogues on the rectus muscle is therefore entirely due to cholinesterase inhibition. As on the diaphragm the effect on cholinesterase of the normal muscle is obscured by a blocking action exerted on the endplates. As regards the intensity of this blocking action the neostigmine analogues can be grouped thus : Ro 2–2783>Ro 2–2650>Ro 2–3198>Ro 2–2651.

Action of neostigmine analogues on avian muscle

Since the experiments on the frog muscle gave very little evidence for an acetylcholine-like action by the neostigmine analogues, innervated and denervated gastrocnemius muscles of the hen were used to study such a property. It was found that, taking the stimulant action of neostigmine as 1, the neostigmine analogues had a direct stimulating property which was of the following order : Ro 2–2650, 1/3 ; Ro 2–2783, 1/3 ; Ro 2–3198, 1/2 ; and Ro 2–2651, 1/30. This stimulating effect is shown neither on the diaphragm nor on the frog rectus, but could occur in the intact animal.

Action on isolated rat intestine

To examine the effects of the neostigmine analogues on a structure where acetylcholine exerts its muscarinic type of action the isolated rat intestine was used.

As can be seen from Table II the neostigmine analogues, with the exception of Ro 2–2783, potentiate the actions of acetylcholine. The potency of the compounds studied was in the order : Ro 2–2651>Ro 2–3198>Ro 2–2650. Whereas Ro 2–2783 and Ro 2–2650 had no effect on the resting tone of the intestine, Ro 2–3198 caused a slight increase and with Ro 2–2651 this was most marked. Neostigmine sensitized the intestine to acetylcholine to a much greater extent than its analogues and spontaneous peristalsis was seen with concentrations of 0.1 µg./ml. and higher ; this was never observed with the neostigmine analogues.

TABLE II

Effect of neostigmine and its analogues on the sensitivity of the isolated rat intestine to acetylcholine. The response to 0.05 μg./ml. Ach is taken as 1 and changes in sensitivity are expressed in multiples of it. 0.05 μg. acetylcholine/ml. was used throughout the whole experiment

Concentration	5×10^{-9}	5×10^{-8}	5×10^{-7}	5×10^{-6}	5×10^{-4}
Ro 2–2650 ..	—	—	1.5	2.4	1/10
Ro 2–2651 ..	—	—	3.6	5.6	5.6 (increase in tone)
Ro 2–2783 ..	—	—	—	1/2	<1/10
Ro 2–3198 ..	—	—	2.5	2/3	<1/5
Neostigmine ..	4.5	6.5 (peristalsis)	—	—	—

Inhibition of cholinesterase in vitro

In Table III the anticholinesterase activities of the neostigmine analogues are summarized. It will be seen that they are of the order of 1/100th to 1/400th of that of neostigmine. The inhibition is both competitive and reversible. If the substrate is added after the inhibitor a constant rate of hydrolysis is reached much earlier with the neostigmine analogues than with neostigmine itself.

TABLE III

Inhibition of red cell esterase by neostigmine and its analogues at 37° C. The activity of cholinesterase during the first 30 min. after addition of the substrate (acetyl-β-methylcholine, 0.03 M.) is taken as a measurement of enzyme inhibition

Inhibitor	Concentration (w/v) which produces 50% inhibition	Relative anticholinesterase potency (neostigmine = 1)
Neostigmine	3.8×10^{-8}	1
Ro 2–2650	1.4×10^{-5}	1/370
Ro 2–2651	6×10^{-6}	1/160
Ro 2–2783	1.1×10^{-5}	1/290
Ro 2–3198	4×10^{-6}	1/110

The neostigmine analogues (except Ro 2–3198) are unstable in aqueous solutions and are split by both cholinesterases I and II. The enzymic hydrolysis is fastest with Ro 2–2651, which is split by cholinesterase II at the same rate as acetylcholine (Table IV). This rate of enzymatic hydrolysis makes it very likely that the actions of Ro 2–2650, Ro 2–2783, and especially of Ro 2–2651 on isolated organs are due to the hydrolysis products, as well as to the esters.

Inhibition of cholinesterase in rabbits in vivo

The neostigmine analogues disappear much more rapidly from the blood stream than neostigmine. With sublethal doses no inhibition of blood cholinesterases was noticeable fifteen minutes after i.v. injections of the substances, whereas the effect of an equiactive dose of neostigmine lasted up to one and a half hours.

TABLE IV

Spontaneous and enzymic hydrolysis at 37° C. in 0.02 M-$NaHCO_3$, as determined by the production of CO_2. Volume: 3 ml. The figures give the output of CO_2 in μl./10 min. The figures for enzymic hydrolysis have been corrected for non-enzymic hydrolysis

Substrate	Spontaneous hydrolysis		Hydrolysis by			
			0.3 ml. (1 : 5 dil.) red cells		0.2 ml. serum	
	0.6 g./ 100 ml.	0.2 g./ 100 ml.	0.6 g./ 100 ml.	0.2 g./ 100 ml.	0.6 g./ 100 ml.	0.2 g./ 100 ml.
Acetylcholine	5.79	2.72	53.64	75.61	109.06	93.15
Acetyl-β-methyl-choline ..	1.98	1.96	51.81	49.68	2.72	2.38
Benzoylcholine	2.64	1.57	1.92	2.94	40.69	42.55
Ro 2–2650	16.48	5.95	13.80	8.92	5.20	3.34
Ro 2–2783	7.22	3.23	15.11	10.03	18.87	10.03
Ro 2–2651	19.89	9.40	47.74	30.38	103.80	70.25

Discussion

The neostigmine analogues Ro 2–2650, Ro 2–2651, Ro 2–2783, and Ro 2–3198 are powerful anticurare agents in cats. Their potencies are of the order of 1/5th to 1/15th of that of neostigmine. Side effects, e.g. salivation, diarrhoea, etc., are either negligible or completely absent. These results confirm fully the experiments of Randall (1950) and Randall and Lehmann (1950). Because of the low anticholinesterase activity *in vitro* (1/100th–1/400th of that of neostigmine) it was originally thought that the mechanism of their anticurare action was not related to cholinesterase inhibition (Randall, 1950 ; Ricker *et al.*, 1949 ; Wescoe *et al.*, 1949 ; Artusio *et al.*, 1950 ; and McFarlane *et al.*, 1950). The experiments reported here, however, show that it is impossible to draw far-reaching conclusions from the *in vitro* experiments.

In studying the anticurare action of drugs with the physico-chemical properties of the neostigmine analogues, we have to consider that their actions could be due to one or more of the following well-established mechanisms :

(1) Inhibition of cholinesterase.

(2) Direct action on the endplate (depolarization).

(3) Inactivation of *d*-tubocurarine.

(4) An action due to their phenolic groups (Mogey and Young, 1949).

Experiments on intact animals do not enable us to determine how much each of these four mechanisms contributes to the anticurare action. This difficulty can be partly overcome by using the isolated phrenic nerve diaphragm and the frog rectus, but from the above experiments it can be seen that each of the two isolated preparations has its limitations : (*a*) The anticurare effect of substances which stimulate the endplate (depolarization) does not show up on the isolated diaphragm. (*b*) The frog rectus does not give a contracture with all substances which can stimulate mammalian muscle (if brought into contact with the endplates suddenly).

Taking into account these limitations the conclusions which can be drawn from the experiments with the neostigmine analogues are as follows :

(1) The neostigmine analogues do not act by combination with *d*-tubocurarine itself.

(2) They do not act by their phenolic nature. The hydrolysis of Ro 2–2650 and Ro 2–2783, whether spontaneous or enzymic, is so slow that most of the actions of these compounds *in vivo* must be produced by the esters and not by their hydrolysis products. Ro 2–2651, however, is rapidly hydrolysed by cholinesterases, especially by cholinesterase II, and part of the action of this compound *in vivo* is certainly due to the hydrolysis product. The free phenolic group in Ro 2–3198, or in the hydrolysis products of the other three esters, is not responsible for the anticurare action as shown on the diaphragm. Whereas phenols, e.g. catechol, have the same anticurare action on a normal diaphragm as on a diaphragm which has lost all its cholinesterase activity by treatment with DFP, the neostigmine analogues, like neostigmine itself, exert no anticurare action on a muscle structure which has been deprived of its cholinesterase.

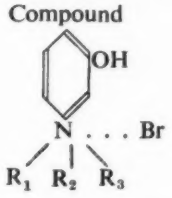
(3) The main mechanism by which the neostigmine analogues act seems to be the inhibition of cholinesterase. On a normal preparation Ro 2–3198 and Ro 2–2651 produce all the symptoms which one would expect from an anticholinesterase, e.g. potentiation of twitch tension, repetitive firing, and sensitization to acetylcholine. With Ro 2–2783 and Ro 2–2651 these effects are obscured by a component which causes neuromuscular block. On a curarized preparation this "second" component is suppressed and the effect of cholinesterase inhibition becomes apparent, but, as one would expect, Ro 2–3198 and Ro 2–2651 are more potent anticurare agents than Ro 2–2650 or Ro 2–2783. On all three preparations (the cat's tibialis muscle, the diaphragm, and the frog rectus) inhibition of cholinesterase completely abolishes the anticurare action, whereas substances with an acetylcholine-like action still act (except on the diaphragm). The rapid onset of the actions of these compounds must be due to a fast rate of penetration as shown by the time necessary to achieve a maximum action with a given concentration on isolated organs. The short duration of action is due to rapid removal of these substances from the circulation.

(4) It is difficult to say how much an acetylcholine-like action is responsible for the anticurare activity of the neostigmine analogues. The anticurare properties of acetylcholine were first described by Wilson and Wright (1937). Recently Hutter (1951) demonstrated similar properties for choline and decamethonium. In my experiments I have confirmed these findings and have also shown that these substances still act in the cat after inhibition of cholinesterase. On the other hand, anticholinesterases and neostigmine analogues lose all their anticurare properties on a preparation deprived of its cholinesterase and some of them even potentiate the blocking action of tubocurarine. Unfortunately the diaphragm is unsuited for the study of the anticurare action of substances with an acetylcholine-like action and all that can be seen on this preparation is a further speeding up of the curarization. On the frog's rectus the neostigmine analogues have practically no stimulant action, and after inhibition of cholinesterase a marked neuromuscular blocking component can be demonstrated. Close arterial injection in cats (Randall and

Lehmann, 1950) and the experiments on avian muscle reveal a weak acetylcholine-like property in these neostigmine analogues, but as the experiments on the cat, after administration of an anticholinesterase, indicate this could be only of minor importance in determining the anticurare action. This view is well supported by Randall's (1950) observation on a series of 3-hydroxy anilinium salts in which one or more methyl groups had been replaced by ethyl groups. Whereas the effective anticurare dose varied only slightly, there was no correlation between acetylcholine-like action and anticurare action (Table V).

TABLE V

Comparison of the anticurare potency and direct action of a homologues series of 3-hydroxy anilinium salts. The data are based on the results given by Randall (1950)

Compound 			Equiactive anti-curare dose in dogs in mg./kg. A	Equiactive dose on denervated muscle in mg. B	Ratio $\frac{A}{B}$
CH_3	CH_3	CH_3	0.2	0.0025	80
CH_3	CH_3	C_2H_5	0.2	0.01	20
CH_3	C_2H_5	C_2H_5	0.3	0.05	6
C_2H_5	C_2H_5	C_2H_5	0.6	>1.00	<0.6

SUMMARY

1. Four neostigmine analogues Ro 2–2650, Ro 2–2651, Ro 2–2783, and Ro 2–3198 have 1/5th–1/15th of the anticurare potency of neostigmine in cats. Their anticholinesterase activity *in vitro* is 1/100th–1/400th of that of neostigmine. The muscarinic side effects are either feeble or absent.

2. The underlying mechanism of their anticurare action seems to be the inhibition of cholinesterase. This has been demonstrated on the tibialis muscle of the cat, on the rat diaphragm, and on the frog rectus abdominis. On each preparation the anticurare action is abolished by previous inhibition of cholinesterase. In non-curarized preparations the manifestations of cholinesterase inhibition can be clearly shown with Ro 2–2651 and Ro 2–3198, whereas with Ro 2–2650 and Ro 2–2783 an additional component, which causes neuromuscular block, interferes with it.

3. An acetylcholine-like action of these compounds on the endplate itself was demonstrated, but seems to be only of minor importance for the anticurare action.

4. An anticurare action by the phenolic groups of these compounds or direct combination with *d*-tubocurarine has been excluded.

I should like to express my thanks to Dr. E. Zaimis, who did the experiments on the hen, and to Dr. J. A. Aeschlimann, Director of Chemical Research, Hoffman–La Roche, Inc., Nutley, New Jersey, U.S.A., for a generous supply of the neostigmine analogues. To Dr. C. A. Keele I am grateful for his very useful discussions and the interest he has shown in this work.

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ON THE ABSENCE AND THE RETURN OF PENDULUM MOVEMENTS AFTER CONTRACTION

BY

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In the course of experiments concerning the origin of the rhythmic spontaneous movements (pendulum movements) of the surviving intestine, it was noted that they were absent or much reduced after large doses of acetylcholine.

Cantoni and Eastman (1946) found a similar decrease in the spontaneous activity (if present previously) of guinea-pig's ileum after large doses of some stimulating drugs (histamine, acetylcholine, mecholyl, pilocarpine, barium chloride), together with a decreased sensitivity to small doses of these drugs. The decrease in sensitivity was not specific for the drug by which the large contraction was elicited, i.e. the response to small doses of histamine was equally depressed by a previous large dose of histamine or pilocarpine. Repeated washings with fresh Tyrode did not shorten the period of decreased sensitivity. The effect of small histamine doses was, however, not diminished after a large contraction elicited by potassium chloride, and if the Tyrode solution contained twice the normal amount of potassium the depressant action of a large dose of histamine was prevented.

Feldberg and Schilf (1930) and Barsoum and Gaddum (1935) showed that small doses of histamine became ineffective after a large dose. Feldberg and Solandt (1942–3) also observed the reduced effect of acetylcholine on rabbit's intestine in glucose-free Tyrode after a large dose of acetylcholine, or if the interval between consecutive acetylcholine additions was shortened.

The depressant after-effect of acetylcholine on striated muscle was described by Gasser and Dale (1926), who found that after a large dose of acetylcholine denervated mammalian muscle may become completely inexcitable electrically. Brown (1937) obtained similar results both with denervated cat's gastrocnemius and with normal and denervated frog's muscle.

That acetylcholine itself can, according to circumstances and dose, produce both excitation and inhibition on the same preparation is also well known (McDowall, 1945, 1946 ; Hoffman, Hoffman, Middleton, and Talesnik, 1945 ; Burn and Bülbring, 1949 ; Burn and Vane, 1949). Burn and Vane (1949) showed on surviving rabbit's intestine that, whereas the usual acetylcholine doses had the well-known contracting effect, a very large dose (about 300 μg. per ml.) added at the height of such a contraction caused a relaxation. Of particular interest is their observation that on

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rat's uterus smaller doses of acetylcholine cause a contraction and large doses a relaxation. After repeated large doses of acetylcholine, however, a smaller dose, that previously caused excitation, causes an inhibition. This action is, however, different from the one studied in this paper. In the experiments to be described the inhibition appeared only after the removal of acetylcholine from the bath, and the dose of acetylcholine given was well within the range causing contraction of the intestine (1–10 μg. per ml.).

Methods

The lowest part of rabbits' ileum was used throughout these experiments, as this shows the most regular pendulum movements when suspended in Tyrode solution *in vitro*. The intestine was removed and used immediately after killing the rabbit by a blow on the head, no experiment being made on stored preparations. Each ileum preparation was 2–4 cm. long and was suspended in a 50 ml. bath containing freshly made Tyrode solution (0.8 g. NaCl, 0.02 g. KCl, 0.02 g. $CaCl_2$, 0.01 g. $MgCl_2$, 0.05 g. NaH_2PO_4, 0.1 g. glucose, 0.1 g. $NaHCO_3$ per 100 ml.) aerated with a mixture of 95% O_2 + 5% CO_2. The temperature of the bath was kept constant at 38.5° C. The contractions of four such preparations were recorded simultaneously, representing thus about 10–15 cm. of the rabbit ileum from the ileocolic sphincter upwards. All preparations immediately showed vigorous and regular pendulum movements, but 20–30 minutes were always allowed (the Tyrode being exchanged three times) before the first addition of drugs. Acetylcholine was usually used. The dose was always given in a volume of 1 ml. by means of a syringe and was left in the bath for different lengths of time. The Tyrode containing this acetylcholine was then let out and the intestine washed twice in quick succession so as to ensure complete removal of the drug.

Experimental Results

Examples of the inhibition following an acetylcholine (ACh) contraction are given in Figs. 1, 2, 4, and 5. The removal of the acetylcholine was followed by an "inhibition," by which term the complete cessation or decreased size of the pendulum movements is meant. In Fig. 1, for instance, 500 μg. acetylcholine was left on the intestine for ten minutes. When the drug was washed out hardly any pendulum movements occurred for about three minutes and then they gradually increased in size. The length of the inhibition varied a great deal with preparations from different rabbits and, to a smaller extent, with the four preparations coming

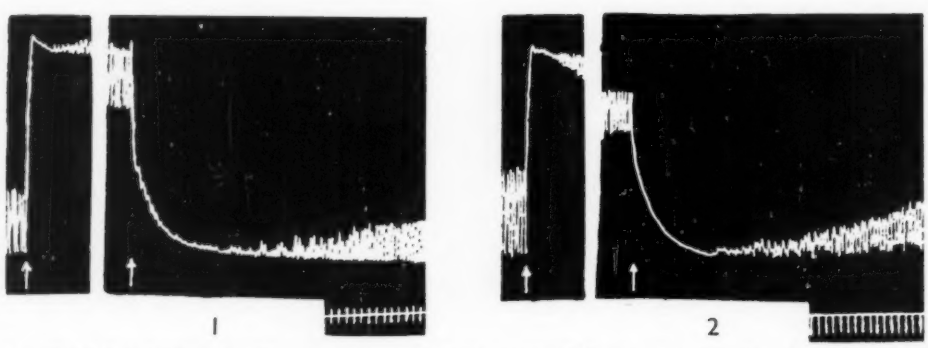

Fig. 1.—Rabbit intestine in 50 ml. bath. At first arrow 500 μg. acetylcholine added and left for 10 min. At second arrow intestine washed with fresh Tyrode. First tracing: at the beginning of the experiment. Second tracing: 4.5 hr. later. Time: 10 sec.

from the same rabbit. On the same intestinal preparation 4–5 consecutive acetylcholine contractions and inhibitions can well be compared with each other and show a remarkable consistency ; e.g. in Fig. 1 the second inhibition shown occurred 4.5 hours after the first, various drugs having been added in between and the acetylcholine addition repeated seven times. On other occasions a gradual lengthening of the inhibition took place, as for instance in the example given in Fig. 2. Two hours elapsed between the different parts of the Figure, and during that time the same dose of acetylcholine was repeated four times (not shown in the Figure).

The inhibition is longer after the removal of larger doses of acetylcholine than after smaller doses. This point is illustrated in Table I. The dose of acetylcholine

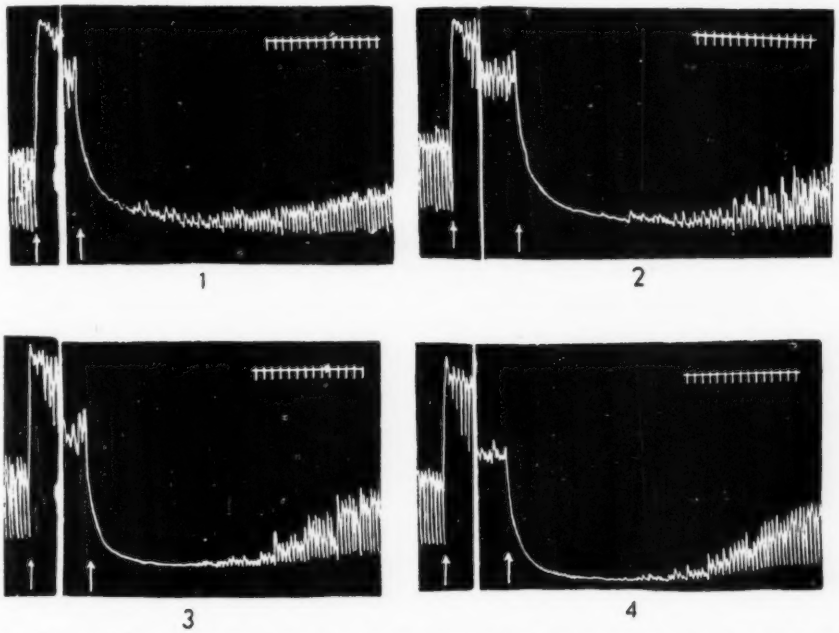

FIG. 2.—Rabbit intestine in 50 ml. bath. At first arrow: 100 μg. acetylcholine for 10 min.; washed at second arrow. First tracing: 0 hr.; second tracing: 2 hr. later; third tracing: 4 hr. later; fourth tracing: 6 hr. later. Time : 10 sec.

TABLE I

THE LENGTH OF THE INHIBITION AS A FUNCTION OF THE ACETYLCHOLINE DOSE

Dose of ACh in μg.	Length of inhibition in minutes and seconds. ACh was left on the intestine for				
	10 min.	10 min.	10 min.	10 min.	5 min.
1	30 sec.				0 sec.
5	2 min. 30 sec.	2 min. 10 sec.			15 sec.
10	3 min. 0 sec.	3 min. 45 sec.			30 sec.
50	4 min. 0 sec.	5 min. 15 sec.	1 min. 15 sec.	4 min. 0 sec.	1 min. 0 sec.
100	4 min. 0 sec.	6 min. 0 sec.	1 min. 40 sec.		1 min. 40 sec.
200			3 min. 20 sec.		
500			5 min. 35 sec.	6 min. 50 sec.	
1,000			5 min. 30 sec.		

was varied from 1 μg. to 1,000 μg. The five columns represent results from intestines of different rabbits. Each dose of acetylcholine was left on the intestine for ten minutes in the first four sets of results and for five minutes in the last. The length of the inhibition is expressed by measuring the time from the washing off of the acetylcholine to the point at which the pendulum movements regained their original size (i.e. that before the acetylcholine addition). It can be seen from the Table that the time taken by the pendulum movements to regain this original size is longer after larger doses of acetylcholine. It can also be seen that there is a great difference between individual rabbits, e.g. the inhibition after ten minutes of 100 μg. acetylcholine varies from 1 min. 40 sec. to 6 min.

TABLE II

THE LENGTH OF THE INHIBITION AS A FUNCTION OF THE LENGTH OF THE ACETYLCHOLINE ACTION

Time of ACh action in minutes	Length of inhibition in minutes and seconds Dose of acetylcholine				
	50 μg.	50 μg.	100 μg.	100 μg.	1,000 μg.
1		45 sec.	20 sec.	30 sec.	25 sec.
2	1 min. 15 sec.				
3		1 min. 45 sec.	1 min. 15 sec.	1 min. 0 sec.	1 min. 0 sec.
4	2 min. 55 sec.				
6		2 min. 15 sec.	1 min. 50 sec.	3 min. 25 sec.	3 min. 45 sec.
8	3 min. 35 sec.				
10		4 min. 15 sec.	2 min. 0 sec.	6 min. 0 sec.	5 min. 50 sec.
16	4 min. 30 sec.		1 min. 50 sec.		
20					5 min. 40 sec.

Table II shows that after a fixed dose of acetylcholine the inhibition lasts longer, the longer the drug has been in contact with the intestine. There are again great individual variations in different rabbits ; for instance, in one rabbit a dose of 100 μg. acetylcholine was not followed by a longer inhibition than two minutes, however long it had been left on the intestine, whereas in other rabbits 50 μg. acetylcholine, acting for 2–4 min., caused an inhibition of similar length.

The absence or decrease in pendulum movements after a large dose of acetylcholine is accompanied by a decreased sensitivity to small doses of other drugs. Shortly after a large dose of acetylcholine the effect of a small dose (e.g. 1 μg.) is much reduced and becomes progressively larger as the pendulum movements reappear and increase in size. This is in agreement with the results of earlier investigators.

As stated by Cantoni and Eastman (1946), the decrease in sensitivity is not specific, i.e. during the inhibition following an acetylcholine contraction the effects of small doses of acetylcholine, histamine, barium chloride, etc., are equally reduced. The lack of pendulum movements and decreased sensitivity to other drugs is, however, not due to the intestine being exhausted at this stage. This was proved in two ways : (1) If the same large dose of acetylcholine (e.g. 100 or 500 μg.) that caused an inhibition is re-added several times during the inhibition, the same contraction is obtained as on the first addition. (2) When, for instance, a dose of 100 μg. acetylcholine is allowed to act for 10 min. and then washed off inhibition appears. If,

however, the same dose of acetylcholine is left on the same intestine for 30–50 min. the intestine goes on showing rhythmic contractions and relaxations, on a higher tone level, without any signs of fatigue and the inhibition only appears when the acetylcholine is exchanged for fresh Tyrode.

The inhibition of the pendulum movements can be observed after all large and lasting contractions that are followed by a sharp relaxation. Thus it was noted after choline (5–100 mg. per 50 ml.), histamine (5–100 mg. per 50 ml.), barium chloride (about 10 mg. per 50 ml.), pilocarpine (10–100 μg. per 50 ml.), and probably even after potassium chloride (1–100 mg. per 50 ml.). Frequently, however, when these drugs are removed there is no immediate relaxation, but only a gradual return to the original relaxed level; this was noted after barium chloride, pilocarpine, and particularly after potassium chloride. Therefore, especially with the last-named drug, it is difficult to say whether there is any inhibition and even more difficult to measure its length. On the other hand, the removal of acetylcholine, however large the dose and however long it has acted on the intestine, is always followed by an immediate and sharp relaxation to the base line. Therefore, acetylcholine has been our drug of preference and was used throughout in attempts to discover the nature of this secondary inhibition.

The inhibition appearing after a large dose of acetylcholine is not due to the liberation into the bath of effective concentrations of some inhibitory humoral agent (e.g. adrenaline). This is shown by the following experiments.

(1) The inhibition is not shortened by exchanging the Tyrode several times during this period, as also observed by Cantoni and Eastman (1946).

(2) The inhibition cannot be transmitted. When one intestinal preparation was completely quiescent after the removal of a large dose of acetylcholine, i.e. while there were no pendulum movements, the Tyrode was sucked off and transferred to another preparation showing normal pendulum movements. The pendulum movements of this second preparation went on uninfluenced by the fluid in which the first preparation had shown no movements at all.

(3) The inhibitions after different doses of acetylcholine were matched with known concentrations of adrenaline and noradrenaline. Ergotoxine, in doses that completely abolished the effect of fifty times these adrenaline or noradrenaline concentrations, had no influence on the inhibition after acetylcholine.

The depression caused by the presence of small doses of acetylcholine on the rat's denervated diaphragm preparation may be relieved by sympathomimetic drugs (adrenaline, ephedrine) and by *d*-tubocurarine (McDowell and Watson, 1951). It seemed interesting to see whether the presence of adrenaline during the contraction would influence the inhibition. It was found that it did not do so as long as it did not alter the acetylcholine contraction itself. It may be mentioned here that the dose of adrenaline that leaves the acetylcholine contraction uninfluenced differs greatly between preparations coming from the ileum and those from the duodenum. The threshold concentrations for both adrenaline and acetylcholine in the duodenum and the ileum of the same rabbit were identical. When the two drugs were mixed, however, an entirely different response was obtained, as seen in Fig. 3. In the duodenum ten times (sometimes even a hundred times) as much adrenaline as acetylcholine can be given together and the result is still a contraction, similar to the one caused by the same dose of acetylcholine alone. In the ileum, on the other hand, the maximal

amount of adrenaline that can be mixed with the acetylcholine without altering the contraction is about a tenth of the acetylcholine dose. If the adrenaline concentration in the mixture is further increased, e.g. adrenaline and acetylcholine are mixed in equal amounts, there is only an inhibition (see Fig. 3).

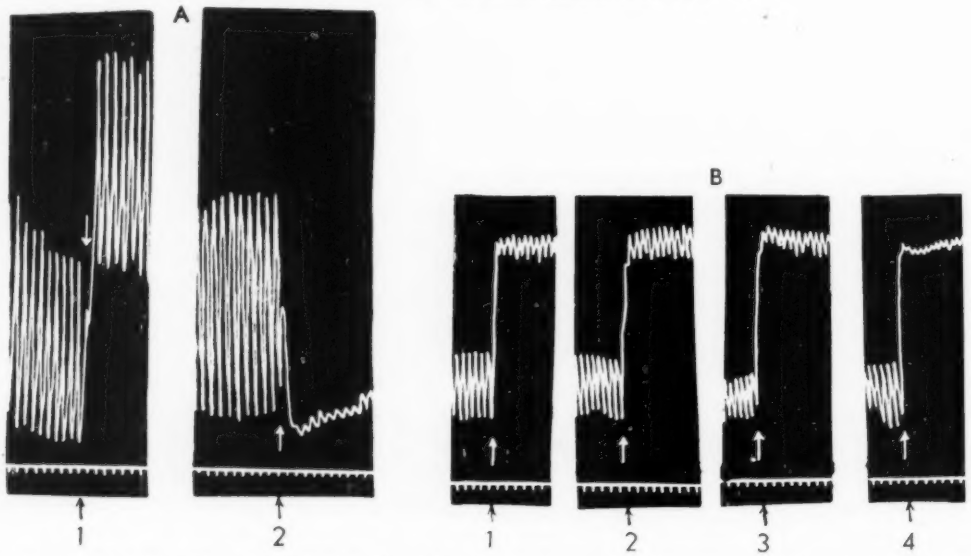

FIG. 3.—Rabbit intestine in 50 ml. bath. A: lower ileum. First arrow: 10 μg. acetylcholine; second arrow: 10 μg. acetylcholine + 10 μg. adrenaline. B: duodenum from same rabbit. First arrow: 10 μg. acetylcholine; second arrow: 10 μg. acetylcholine + 10 μg. adrenaline; third arrow: 10 μg. acetylcholine + 100 μg. adrenaline; fourth arrow: 10 μg. acetylcholine + 1,000 μg. adrenaline. Time: 5 sec.

Drugs which paralyse ganglionic and nervous structures have very little influence on the inhibition following an acetylcholine contraction as long as they do not interfere with the contraction itself.

(1) Hexamethonium, in concentrations of 10^{-7} to 2×10^{-4} has no, or very little, influence either on the pendulum movements, or on the acetylcholine contraction (Feldberg, 1951), or on the inhibition which follows.

(2) *d*-Tubocurarine up to a concentration of 2×10^{-4} does not alter the acetylcholine contraction (Feldberg and Lin, 1949) or the inhibition.

(3) While nicotine (10^{-7} to 2×10^{-4}) hardly alters the contraction (Emmelin and Feldberg, 1947), there may be a slight tendency of the inhibition to become longer; 2×10^{-3} nicotine completely paralyses the intestine, i.e. the pendulum movements disappear and acetylcholine produces no contraction.

(4) The acetylcholine contraction takes place in the presence of cocaine, until the concentration of the latter reaches 2×10^{-3}, when complete paralysis occurs. Below a cocaine concentration of 2×10^{-3} the height of the acetylcholine contraction is hardly altered and the inhibition following the contraction is also unchanged, though there is a tendency for the pendulum movements to become smaller and more irregular with increasing cocaine concentrations (Feldberg and Lin, 1949).

(5) Proguanil acts like nicotine, complete paralysis taking place when its concentration in the Tyrode reaches 2×10^{-4} (Vane, 1949).

The inhibition after an acetylcholine contraction becomes longer if the glucose content of the Tyrode is reduced. After 30 min. in Tyrode containing only half the normal amount of glucose (0.05 g./100 ml.), the pendulum movements and the acetylcholine contraction remained unchanged, but after the acetylcholine contraction, the pendulum movements returned to normal much more slowly, i.e. the inhibition was prolonged ; 30 min. in normal Tyrode completely restored the inhibition to its normal length. The pendulum movements decrease in size during 30 min. in glucose-free Tyrode, the acetylcholine contraction is much reduced, the tone of the contraction not being maintained (Feldberg and Solandt, 1942–3), and when the acetylcholine is removed the pendulum movements do not reappear. When the bath fluid is replaced by normal Tyrode, however, complete recovery takes place.

As a next step the effect of alterations in the ionic composition of the Tyrode was investigated ; that this might influence the inhibition after acetylcholine was indicated by the experiments of Cantoni and Eastman (1946), who found that the inhibition disappeared if the potassium content of the Tyrode was increased to twice its normal value.

After the inhibition following a suitable dose of acetylcholine had been established in normal Tyrode, the bath fluid was replaced by Tyrode solutions containing two, three, or four times (in the case of Mg up to six times) the normal amount of

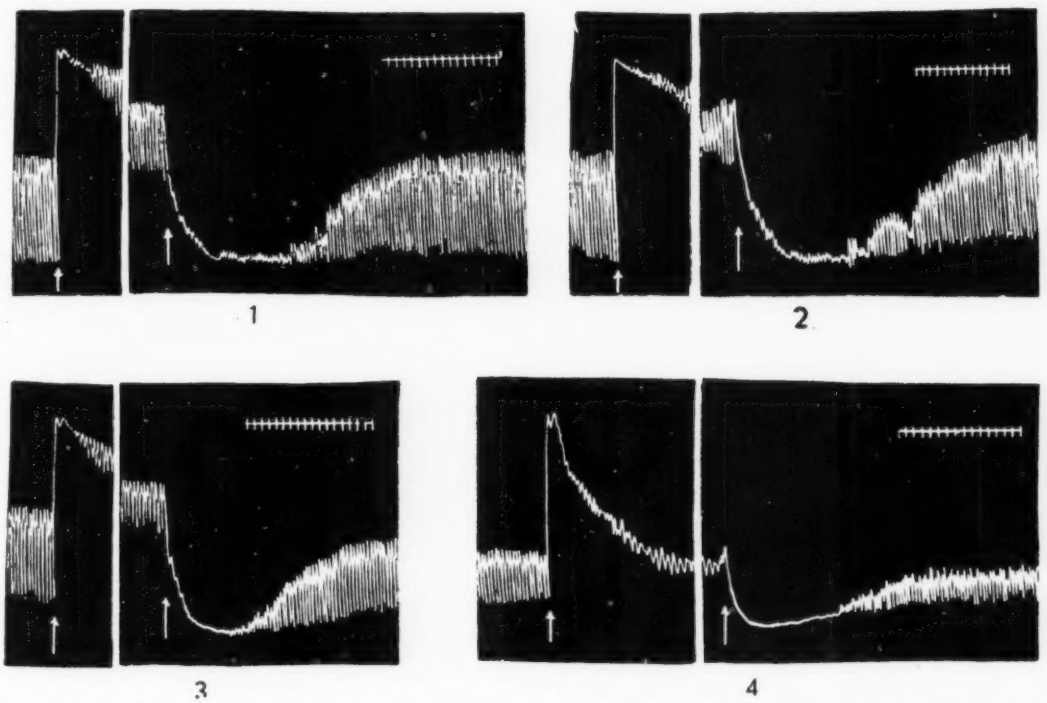

FIG. 4.—Rabbit intestine in 50 ml. bath. In each tracing 500 μg. acetylcholine added at first arrow left for 10 min., and washed out with fresh Tyrode at second arrow. (1) In normal Tyrode. (2) In Tyrode with twice the normal amount of potassium (0.04% KCl). (3) In Tyrode with three times the normal amount of potassium (0.06% KCl). (4) In Tyrode with four times the normal amount of potassium (0.08% KCl). Time: 10 sec.

potassium or calcium or magnesium. The same dose of acetylcholine as before was then added and the intestine was washed with Tyrode of the same increased ionic content as that in which the contraction had occurred. The inhibition after acetylcholine occurred in all solutions tried. Fig. 4 shows the inhibition in Tyrode containing an increased potassium content.

The same experiment was then repeated with Tyrode solutions in which the concentrations of K, Ca, or Mg was reduced to $\frac{1}{2}$, $\frac{1}{3}$, or $\frac{1}{4}$ of the normal. A few experiments were also made with one ion missing altogether from the Tyrode. The inhibition was present in every solution and there was no unequivocal change in its duration, though the inhibition seemed to be somewhat shorter in Tyrode solutions with a reduced potassium content.

The experiment was then varied in such a way that five consecutive acetylcholine contractions and inhibitions formed one set of experiments : (1) Acetylcholine contraction and inhibition in normal Tyrode. (2) Acetylcholine contraction in normal Tyrode, inhibition in Tyrode with altered ionic composition. (3) Both acetylcholine contraction and inhibition in Tyrode with altered ionic composition. (4) Acetylcholine contraction in Tyrode with altered ionic composition, inhibition in normal Tyrode. (5) Both acetylcholine contraction and inhibition in normal Tyrode, i.e. repetition of (1) in order to show that no change had occurred in the meanwhile in the length and strength of the inhibition. If in the above set of experiments the Tyrode with altered ionic composition is Tyrode with increased potassium content, there is no change in the inhibition. If, however, it is Tyrode with reduced ($\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{4}$

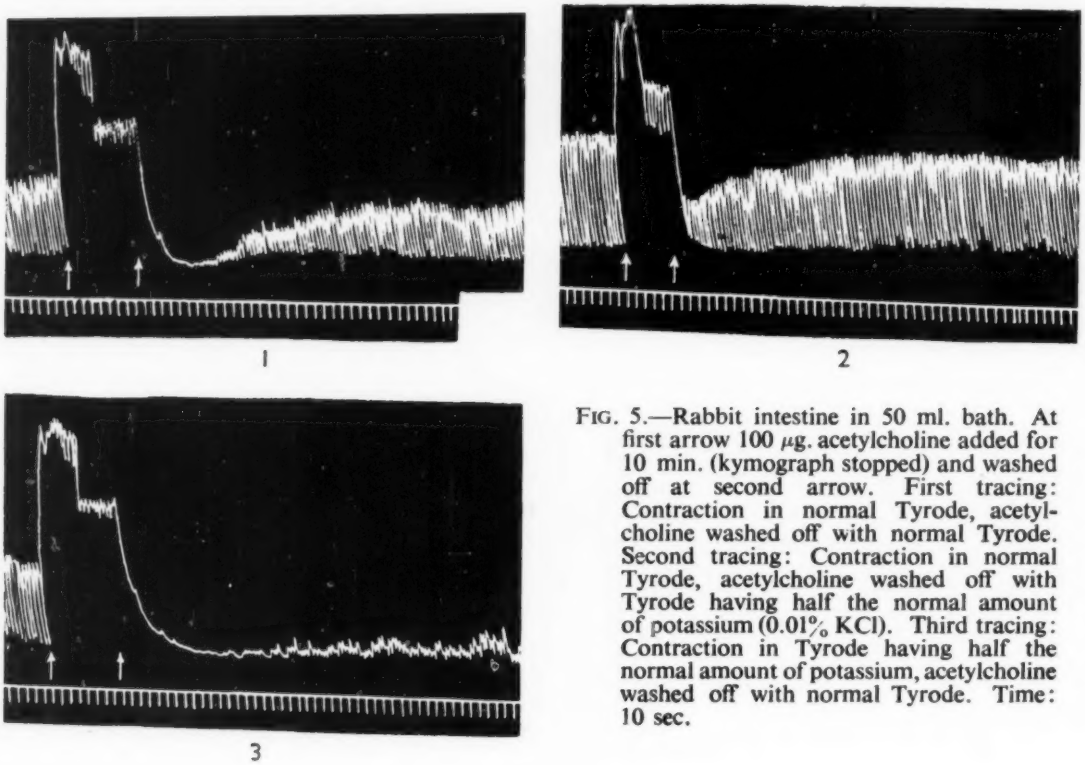

FIG. 5.—Rabbit intestine in 50 ml. bath. At first arrow 100 μg. acetylcholine added for 10 min. (kymograph stopped) and washed off at second arrow. First tracing: Contraction in normal Tyrode, acetylcholine washed off with normal Tyrode. Second tracing: Contraction in normal Tyrode, acetylcholine washed off with Tyrode having half the normal amount of potassium (0.01% KCl). Third tracing: Contraction in Tyrode having half the normal amount of potassium, acetylcholine washed off with normal Tyrode. Time: 10 sec.

normal) potassium content, the inhibition is practically abolished when the acetylcholine is applied in normal Tyrode, but washed out with Tyrode containing a reduced potassium content (Exp. 2 above). On the other hand, the inhibition is much prolonged if the order of the solutions is reversed, i.e. if the acetylcholine is applied in Tyrode with reduced potassium content, but washed out with normal Tyrode (Fig. 5). If, instead of the potassium, the calcium content of the Tyrode is altered, the following results are obtained. When acetylcholine is applied in normal Tyrode, but washed out with Tyrode containing an *increased* calcium content (Exp. 2 above), the inhibition is much prolonged. When acetylcholine is applied in normal Tyrode and washed out with Tyrode containing a *reduced* calcium content, the results are not so unequivocal. In one rabbit we saw an actual shortening of the inhibition ; in all other rabbits, however (8 in all), the inhibition was unaltered or prolonged. Other possible combinations with increased or decreased calcium content had no influence on the inhibition.

Lastly the effects on the pendulum movements of all the above alterations in the ionic composition of the Tyrode were observed in the absence of acetylcholine. The only regular changes observed were the following : There is an inhibition of the pendulum movements (similar to the one seen after an acetylcholine contraction) if normal Tyrode is exchanged for one having an increased calcium content (Fig. 6). An even more pronounced and long-lasting inhibition is observed if the intestine is in Tyrode with reduced potassium content and this is exchanged for normal Tyrode (Fig. 7). No other alteration in the ionic composition of the Tyrode had

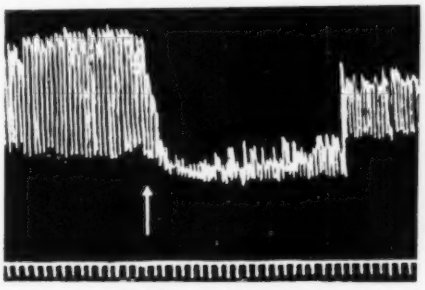

FIG. 6.—Rabbit intestine in 50 ml. bath. At arrow: Normal Tyrode replaced by Tyrode with twice the normal amount of calcium (0.04% $CaCl_2$). Time: 10 sec.

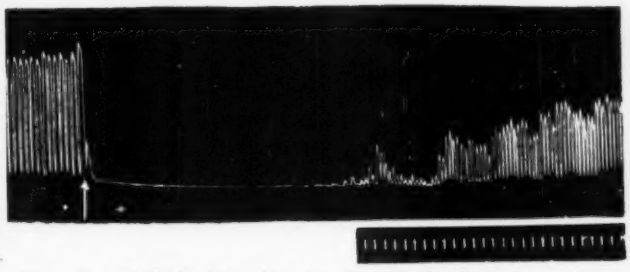

FIG. 7.—Rabbit intestine in 50 ml. bath. At arrow: Tyrode containing one-third the normal potassium content replaced by normal Tyrode. Time: 10 sec.

any marked effect on the pendulum movements. Alterations in the first few beats after the change may occur, but some irregularities also occur when normal Tyrode is simply replaced by fresh normal Tyrode. The one further change that may be mentioned here is that the frequency of the pendulum movements decreased if the calcium content of the Tyrode was decreased.

DISCUSSION

It seems to be well established that, on a surviving intestinal preparation, a period of decreased sensitivity and an inhibition of the pendulum movements follow any large contraction. It also seems to be certain that this inhibition is not specific

to any one drug and is not due to fatigue of the preparation or to the accumulation of some inhibitory substance. So far the results here presented are in agreement with those in the literature.

Cantoni and Eastman (1946) found, however, on the guinea-pig's ileum that if the Tyrode contained twice the normal amount of potassium a large dose of histamine was not followed by a period of decreased sensitivity to small histamine doses. In our experiments on the rabbit's ileum, the inhibition after a large acetylcholine contraction occurred whether the Tyrode contained normal or twice, three, or four times the normal amount of potassium. The cause of this discrepancy is not known. It has to be pointed out, however, that Cantoni and Eastman's results were obtained on the guinea-pig's ileum and the sensitivity was tested after a large histamine dose by the contractions given to small doses of histamine. In the present experiments rabbit's ileum was used and the inhibition of the pendulum movements was determined after a large acetylcholine contraction.

A decrease in the potassium content or any change in the calcium or magnesium content of the Tyrode have likewise no definite influence on the inhibition, if contraction and relaxation occur in a Tyrode having a similar composition.

The following changes in the length of the inhibition after the same large acetylcholine contraction have been regularly observed.

(1) The inhibition is much prolonged if (*a*) the acetylcholine is applied in Tyrode with reduced potassium content and removed with normal Tyrode, or if (*b*) the acetylcholine is applied in normal Tyrode and removed with Tyrode containing an increased calcium content. These same changes, i.e. changing from Tyrode with decreased potassium content to normal Tyrode or changing from normal Tyrode to one with increased calcium content, also cause an inhibition of the pendulum movements themselves, when no acetylcholine has been added. Therefore, it seems likely that the inhibition occurring after an acetylcholine contraction alone and the inhibition caused by these ionic changes simply summate.

(2) The inhibition is much shortened or altogether absent if the acetylcholine contraction takes place in normal Tyrode and the acetylcholine is removed with Tyrode containing reduced potassium content. The picture would be similar, if in some way an acetylcholine contraction reduced the amount of potassium in the Tyrode ; washing with normal Tyrode would then cause an inhibition, even in the absence of any previous contraction.

Decreasing the glucose content of the Tyrode prolongs the inhibition, but this only happens if the intestine has been for some time in the reduced-glucose-Tyrode. Reduction of the glucose content of the Tyrode, i.e. exhaustion of the carbohydrate reserve during a normal acetylcholine contraction, cannot, however, be the cause of the inhibition, because (*a*) the inhibition itself occurs in fresh Tyrode, (*b*) as long as the acetylcholine is left on the intestine, no inhibition appears, and (*c*) there is no inhibition if relaxation takes place in Tyrode with a reduced potassium content.

Summary

1. An acetylcholine contraction of the surviving rabbit's intestine is followed by an inhibition after the removal of the acetylcholine. The length of the inhibition increases with the size and duration of the preceding acetylcholine contraction.

2. The absence or decrease of the pendulum movements during this inhibition runs parallel with a decreased sensitivity of the preparation to other chemical stimuli.

3. The inhibition is not specific to acetylcholine; it is present after all prolonged contractions which are followed by a sharp relaxation.

4. The inhibition is not due to fatigue of the preparation, nor to the liberation of some inhibitory chemical agent. It is uninfluenced by nicotine, hexamethonium, *d*-tubocurarine, cocaine, and proguanil. It is present in Tyrode solution with increased or decreased potassium, calcium, or magnesium content, if both contraction and relaxation take place in identical solutions.

5. The presence of adrenaline during the contraction does not influence the subsequent inhibition as long as it does not alter the contraction itself. The admixture of much more adrenaline is needed to change an acetylcholine contraction into relaxation in the duodenum than in the ileum of the same rabbit.

6. An acetylcholine contraction is not followed by an inhibition if the acetylcholine contraction takes place in normal Tyrode and the acetylcholine is removed with Tyrode containing a reduced potassium content.

7. The inhibition is prolonged (*a*) if the glucose content of the Tyrode is reduced, (*b*) if the acetylcholine contraction takes place in Tyrode with reduced potassium content and the inhibition in normal Tyrode, and (*c*) if the acetylcholine contraction takes place in normal Tyrode and the inhibition in Tyrode with increased calcium content.

8. In the absence of acetylcholine, the pendulum movements are inhibited by (*a*) changing from reduced potassium Tyrode to normal Tyrode and (*b*) changing from normal Tyrode to Tyrode with increased calcium content.

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MECHANISM OF METHIONINE PROTECTION AGAINST THE NEPHROTOXICITY OF POLYMYXIN A

BY

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Of the five polypeptide antibiotics derived from various strains of *Bacillus polymyxa*, one of them, polymyxin A, has a nephrotoxic action in both animals and man (Brownlee, Bushby, and Short, 1949). The kidney damage, as indicated by proteinuria, occurs within twenty-four hours of administration, the peak protein excretion lasting between three and four days, the proteinuria then gradually decreasing in spite of continued dosage with the antibiotic. Cellular and granular casts continue to be excreted occasionally for several weeks after the cessation of treatment.

Histological examination of the kidneys at various stages of treatment in rats, guinea-pigs, and dogs shows that the damage is mainly confined to the convoluted tubules. The progressive effects were followed in dogs given a five-day course of treatment consisting of four three-hourly injections of 1 mg./kg. each day. Three days after the commencement of treatment, the picture was one of acute severe nephrosis with the secretory epithelium in all stages of disintegration and large numbers of cellular, granular, and hyaline casts. Seven days later there was still histological evidence of active damage with generalized cloudy swelling, stripping of the tubular epithelium, some granular and hyaline casts, and patches of severe oedema resulting in blockage of the damaged nephrons. After twenty-one days, there was no evidence of progressive damage ; although many of the nephrons were blocked and apparently non-functional, many others appeared normal.

The similarity of the nephrotoxic action of polymyxin A to that of DL-serine, as described by Artom, Fishman, and Morehead (1945), has already been pointed out (Brownlee and Short, 1949) and confirmation obtained that a similar protection in rats and dogs is obtained with certain amino-acids (Brownlee, Bushby, and Short, 1948) to that reported for serine by Wachstein (1947).

An investigation of the possible mechanisms of the nephrotoxicity and this protection is described here.

METHODS

Assessment of the renal toxicity

In rats.—The resultant proteinuria in rats has been used to evolve a semi-quantitative method for measuring the effect of antagonists on the renal toxicity of polymyxin A. Groups of four rats given graded doses of polymyxin A showed increased amounts of

protein in the urine during 72 hours. The animals were kept in metabolism cages at a constant temperature of 20–22° C. to minimize variations in renal flow due to temperature and were hydrated with 10 ml. water/100 g. rat before dosage. The dose selected as giving the best response was 1 mg./100 g. rat, since higher doses approached too near to the LD50 and lower doses gave uncertain responses. The total weight of protein excreted in 72 hours, expressed as mg. protein/100 g. rat, was compared with that excreted by a group of animals given a standard batch of polymyxin A and a control group given saline. The reproducibility of the test is low, largely because of the variations in the normals.

In dogs.—The effect of continuous treatment with the antibiotic was studied in the dog, since it proved more sensitive in detecting small amounts of damage. The maximum protein concentration in the urine (g./litre) attained in 96 hours was used as a measure of the extent of the renal damage, since it was observed that after this time the degree of proteinuria decreased even when treatment was continued.

Estimation of protein

The protein was precipitated with trichloroacetic acid and then estimated absorptiometrically by means of the biuret reaction in the presence of ethylene glycol. This modification (Mehl, 1945) has the advantage that a clear solution is obtained and centrifuging to remove copper hydroxide is avoided.

Estimation of methionine

The methionine concentration in the urine was determined by a modification of the method described by Albanese, Frankston, and Irby (1944). It depends on the selective oxidation of the sulphydryl group with hydrogen peroxide in the presence of perchloric acid. Samples of urine (15 ml.) were treated with a measured excess of hydrogen peroxide-perchloric acid mixture, and after exactly one hour the excess oxidizing reagent was determined by the addition of potassium iodide and titration of the liberated iodine with 0.1 N-sodium thiosulphate The method is accurate to within ± 2.5 per cent.

Estimation of polymyxin

The concentration of polymyxin in serum and urine was determined microbiologically by the serial dilution method in a glucose medium containing 1 per cent phenol red broth and 50 per cent (v/v) horse serum inoculated with *Bact. coli* (Brownlee and Bushby, 1948).

Results and Discussion

Complete protection in rats against the nephrotoxicity of a dose of 1 mg./100 g. rat of polymyxin A was obtained by treatment with methionine or S-methyl-cysteine. When the antibiotic was given alone, about five times as much protein was excreted by the test group of animals as by the controls, while simultaneous administration of 200 mg. methionine or S-methyl-cysteine per 100 g. rat reduced the weight of protein excreted to values similar to those obtained with the controls. No protective action was shown by the substances containing an unmethylated thiol group, such as L-cysteine and 2 : 3-dithiopropanol.

A similar protective action was obtained with methionine and S-methyl-cysteine in dogs. Table I shows the effect of the two substances on a course of treatment with polymyxin A of 1.0 mg./kg. given by subcutaneous injection, three times daily for three days. The proteinuria was completely prevented by simultaneous treatment with ten times the amount of antagonist in each case. With the lower doses of

TABLE I

PROTECTIVE EFFECT OF METHIONINE AND S-METHYL-CYSTEINE AGAINST NEPHROTOXICITY OF POLYMYXIN A IN DOGS

1 mg./kg. polymyxin A, three times daily for three days

Antagonist	Dose mg./kg.	Max. conc. protein in urine in 96 hours (g./l.)
Methionine	10	0
	5	0, 0.1
	2.5	7.0
S-Methyl-cysteine	10	0
	5	0.3
Nil		5.0, 2.0

antagonist, some proteinuria developed, but urinary deposits were free from renal epithelial cells and casts, and histological examination of the kidneys showed no evidence of stripping but only a cloudy swelling of the epithelial cells.

Wachstein (1947) suggested that the protective effect of methionine and other substances against the renal damage produced by serine in the rat was due to com-

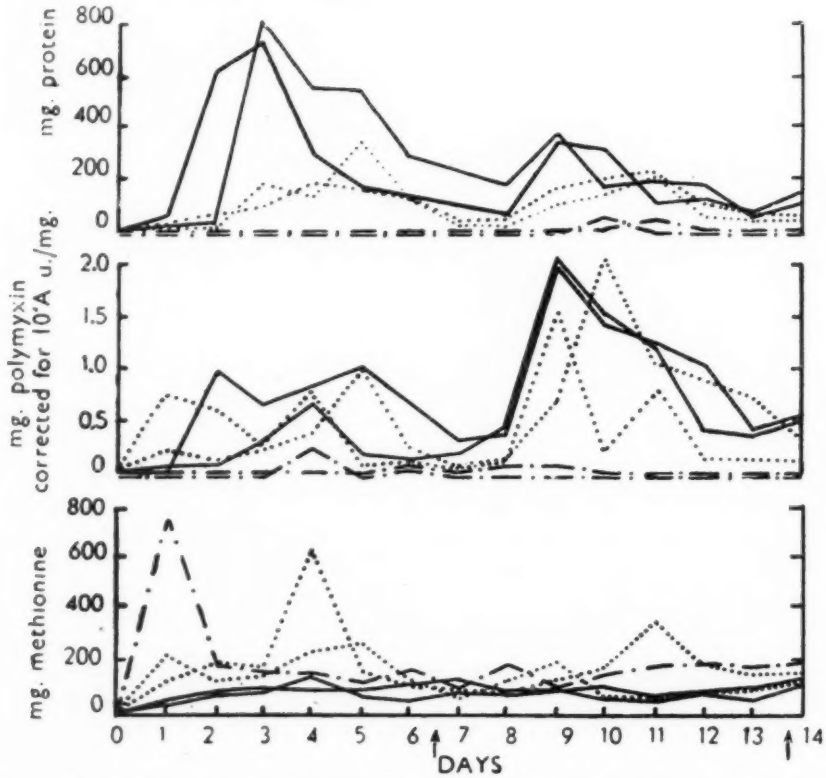

FIG. 1.—Comparison of the excretion of protein, polymyxin A, and methionine in dogs treated three times daily, except at the arrows, with 1 mg./kg. of antibiotic. Continuous lines: polymyxin A alone; dotted lines: with methionine (20 × dose); and dot-dash lines: with methionine (40 × dose).

petitive tubular resorption, the preferential reabsorption of the antagonist preventing the reabsorption of the damaging substance. A study of the comparative excretions of antibiotic, methionine, and the extent of proteinuria does not support this mechanism in the case of polymyxin A. Fig. 1 compares the excretion of protein, polymyxin A, and methionine in dogs treated three times daily with 1 mg./kg. of antibiotic alone and with twenty and forty times the dose of methionine. Treatment was continuous except at the arrows. Contrary to what would be expected if methionine protected entirely by preferential tubular reabsorption, more antibiotic was excreted when proteinuria was present and the excretion of methionine did not run concurrently with the protein excretion. Confirmation is given by the results obtained in a similar experiment with polymyxin B (Fig. 2), which is

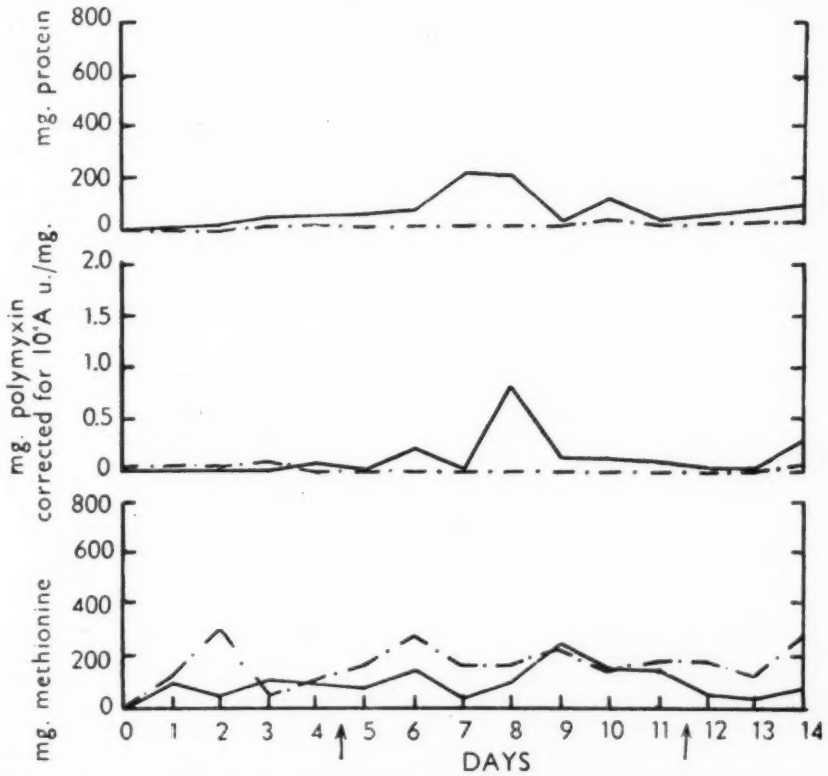

FIG. 2.—Comparison of the excretion of protein, polymyxin B, and methionine in dogs treated continuously, except at the arrows, with three daily doses of 1 mg./kg. Continuous lines: polymyxin B alone; dot-dash lines: with methionine (20 × dose).

practically free from nephrotoxicity. There was practically no proteinuria, and only a negligible excretion of antibiotic and no increase in methionine excretion occurred.

Thus it appears that competitive tubular reabsorption does not alone account for the protective action, and since it is unlikely that glomerular or tubular filtrates would damage specifically the distal tubules, the possibility of detoxication owing to concurrent chemical change accompanying the reabsorption of the toxic substance was investigated.

It is significant that L-cysteine and 2 :3-dithiopropanol gave no protection in the rat, so the reaction cannot be associated with the sulphydryl groups of methionine and S-methyl-cysteine.

Methionine contains a readily utilizable methyl group and is known to be capable of replacing choline as a transmethylating agent. Also S-methyl-cysteine, which gives as good protection as methionine, contains a labile methyl group. The possibility that polymyxin A produces its nephrotoxicity by an acquisition of labile methyl groups and that the protective mechanism is one of supplying such groups is thus indicated.

The effect of a number of compounds containing methyl groups was determined. The results given in Table II show that the two compounds which are known to

TABLE II

EFFECT OF METHYLATING COMPOUNDS AS PROTECTIVE AGENTS IN RATS

Dose of polymyxin A, 1.0 mg./100 g. rat

Substance	Dose mg./100 g. rat	Wt. protein excreted in 72 hours, mg./100 g. rat
Choline	50	24.7, 30.0
	200	9.2
Methyl isothiourea	50	18.2
	100	11.4
Caffeine sodium benzoate	10	48.0, 51.5
	20	32.5, 45.2
	40	22.5, 28.6
Theophylline sodium acetate	5	31.2
	10	38.3
	20	15.6
Nil		55.5, 56.3, 61.5
Controls		5.4, 10.7, 14.5

be active transmethylating agents, choline and S-methyl-thiourea, gave complete protection. The methyl-xanthine derivatives also gave some protection at the highest of the dose levels used.

Finally, it is significant that the nephrotoxicities of the acetyl derivative of polymyxin A and the product of the reaction of polymyxin A with diazomethane (probably methyl polymyxin) are less than that of the parent substance. The acetyl derivative had 12.3 per cent residual activity and the methyl compound 45.5 per cent. Given in doses equivalent to the activity of 1 mg. of the original antibiotic per 100 g. rat, complete protection was obtained in both cases (Table III).

It has been observed that, during a five-day course of treatment with the antibiotic in man, the peak proteinuria developed at three and a half to four days and was resolving on the fifth day, even though dosage was continued until the fifth day (Brownlee, Bushby, and Short, 1949). This phenomenon was studied in dogs which were treated continuously with 1 mg. polymyxin A per kg., three times daily for three weeks. The development of proteinuria is shown in Fig. 3. A first peak, similar to that obtained in man, developed between the third and fourth day of treatment, and a second smaller peak between the ninth and tenth day, after which, in spite of continued treatment, only low concentrations of protein were found in

TABLE III

RENAL TOXICITY OF POLYMYXIN A DERIVATIVES

Doses equivalent to 1 mg. polymyxin A/100 g. rat

Substance	Wt. protein excreted in 72 hours mg./100 g. rat
Methylated polymyxin A	13.1, 6.1
Acetylated polymyxin A	5.1, 5.8
Polymyxin A	43.5, 41.7
Controls	5.7, 7.6, 14.5

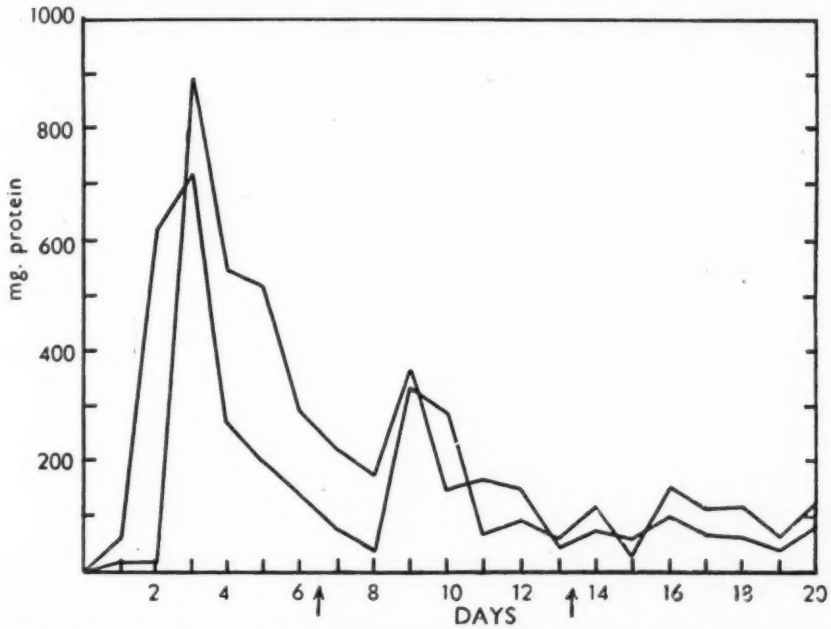

FIG. 3.—Proteinuria in dogs treated for three weeks with three daily injections of polymyxin A, 1 mg./kg.

the urine. These results, together with the histological evidence of the failure of the lesions to progress, suggest that the body is able to mobilize a defensive mechanism similar to that afforded by methionine and other methylating agents.

SUMMARY

1. Polymyxin A produces renal damage in both experimental animals and man.

2. The mechanism of the nephrotoxicity and of the action of certain antagonists has been studied by semi-quantitative methods in rats and dogs based on estimates of the resultant proteinuria.

3. DL-Methionine and S-methyl-L-cysteine in sufficient dosage give complete protection against the nephrotoxicity of polymyxin A in rats and dogs.

4. Comparison of the excretion of protein, antibiotic, and methionine after treatment with polymyxin A and polymyxin B, which has only negligible nephrotoxicity,

indicated that the protective action was not due simply to competitive tubular resorption.

5. Methionine and S-methyl-cysteine both contain labile methyl groups, and two other transmethylating agents, choline and S-methyl-thiourea, also gave good protection in rats.

6. The acetyl and methyl derivatives of polymyxin A did not show any nephrotoxicity.

7. It is concluded that the nephrotoxicity of polymyxin A is due to a drainage of methyl groups and that antagonists produce protection by supplying these groups.

8. There is some evidence that the body mobilizes a defence mechanism which may be analogous to the protection given by methylating compounds, since in both animals and man the peak proteinuria occurs between the third and fourth day of treatment, after which the amount of protein excreted diminishes in spite of continued dosage with the antibiotic.

I wish to thank Mr. S. R. M. Bushby for the estimations of polymyxin, Dr. S. Wilkinson for the preparation of the acetyl and methyl derivatives of polymyxin A, and Dr. D. Trevan for the histological preparations of the kidneys.

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EFFECT OF HEXAMETHONIUM ON THE RESPONSE TO INSULIN IN ANIMALS AND MAN

BY

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The work described below was planned to investigate two aspects of the action of hexamethonium in relation to insulin hypoglycaemia.

Cannon, McIver, and Bliss (1924) first clearly demonstrated that hypoglycaemia increased sympathetic activity and adrenaline secretion, and that, by the mobilization of glucose from the liver, this forms a protective mechanism against an extreme fall in blood sugar. Hexamethonium, by blocking sympathetic impulses to the liver and adrenal medulla, might be expected to modify the course of hypoglycaemia and by impairing the protective mechanism to lead to a greater fall in blood sugar.

Secondly, we have shown in a previous paper (Laurence and Stacey, 1952) that hexamethonium raises the convulsant dose of nicotine in rats and mice but does not affect that of certain other convulsants. It was thought that the effect of hexamethonium on hypoglycaemic convulsions might help to elucidate this action.

Effect of Hexamethonium on the Insulin Convulsion Rate

Two experiments were done on each of ten rabbits. After 24 hours' starvation 0.8 i.u. insulin per kg. was given intravenously, alone on one occasion, and on the other with 5 mg. per kg. hexamethonium iodide also intravenously. Hexamethonium increased the convulsion rates from 1 in 10 to 7 in 10. Three rabbits were not convulsed in either experiment ; one was convulsed in both, the convulsion in the experiment in which hexamethonium was given being very much more severe. The value of P in this experiment was <0.01 ; it was obtained from Mainland's fourfold contingency tables (Mainland, 1948).

Effect of Hexamethonium on the Course of Insulin Hypoglycaemia

Four human subjects and six rabbits were used. The human subjects (two were normal and two had anxiety states) had fasted for twelve hours, the rabbits for twenty-four hours ; water was allowed. The blood sugar was estimated at ten-minute intervals after a small intravenous injection of insulin. The experiment was repeated on each individual on a subsequent day under the same conditions but with the additional administration of hexamethonium, intramuscularly in the human cases, intravenously in the rabbits. The time relations and dosage are indicated in Table I and Fig. 2. In the human experiments observations were made on the heart rate, blood pressure, sweating, E.E.G., and subjective sensations. "True" blood sugar was estimated by the method of King (1947), using capillary blood in man and venous blood in rabbits.

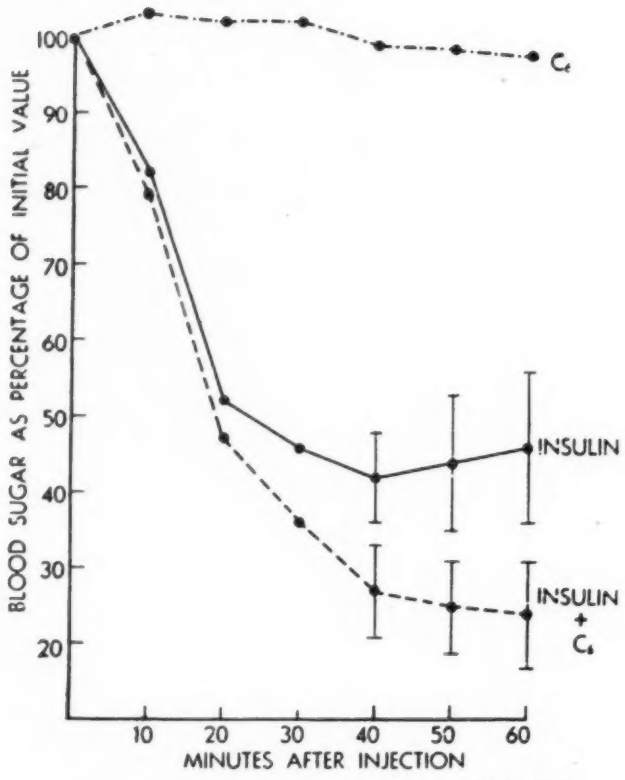

FIG. 1.—Mean blood sugar curves from six fasting rabbits. ●-.-.-● hexamethonium alone (5–10 mg./kg.). ●——● insulin alone (0.8 i.u./kg.). ●- - -● same dose of insulin with hexamethonium (for doses see Table I). Vertical lines indicate the standard deviation. All injections were intravenous.

TABLE I

Blood sugar at 10 min. intervals after insulin alone (ordinary type), and after insulin and hexamethonium iodide (italics) in six rabbits. Dose of insulin: 0.8 i.u./kg. i.v.

Rabbit	Blood sugar, mg./100 ml. blood							Hexamethonium iodide mg./kg. i.v.	Convulsions: present + absent −
	Zero hour	10′	20′	30′	40′	50′	60′		
A	125 *144*	113 *107*	75 *64*	60 *42*	58 *43*	60 *36*	60 *43*	— 5 at 0 min.	— —
B	141 *120*	121 *96*	63 *48*	49 *37*	43 *24*	45 *22*	46 *27*	— 5 at 0 min.	— +
C	93 *98*	63 *82*	43 *44*	35 *31*	37 *23*	38 *19*	41 *22*	— 5 at 0 min.	— +
D	87 *76*	88 *60*	63 *38*	50 *29*	40 *18*	40 *17*	51 *17*	— 5 at 0 min.	— +
E	86 *76*	64 *57*	36 *38*	47 *31*	40 *23*	50 *25*	47 *24*	— 3.3 at 0′, 10′, 20′	— +
F	85 *67*	61 *54*	37 *35*	35 *28*	34 *25*	35 *21*	31 *9*	— 3.3 at 0′, 10′, 20′	— +
Av. S.D. *Av.* *S.D.*	103 *97* 	85 *77* 	53 *45* 	46 *33* 	42 ±8.5 *26* *±8.5*	45 ±9 *23* *±7.5*	46 ±9.5 *24* *±11.5*		

Blood sugar curves

Rabbits.—The results of experiments on six rabbits are shown in Table I and Fig. 1. In every animal the fall in blood sugar was greater when hexamethonium was given with the insulin. During the first twenty minutes the differences in the blood sugar curves were insignificant, but they became more and more marked during the succeeding thirty minutes. In these experiments no rabbits were convulsed by the dose of insulin (0.8 i.u./kg. i.v.) given alone, but five out of six were convulsed when hexamethonium was given with it. Hexamethonium alone (Fig. 1) had little effect on the blood sugar.

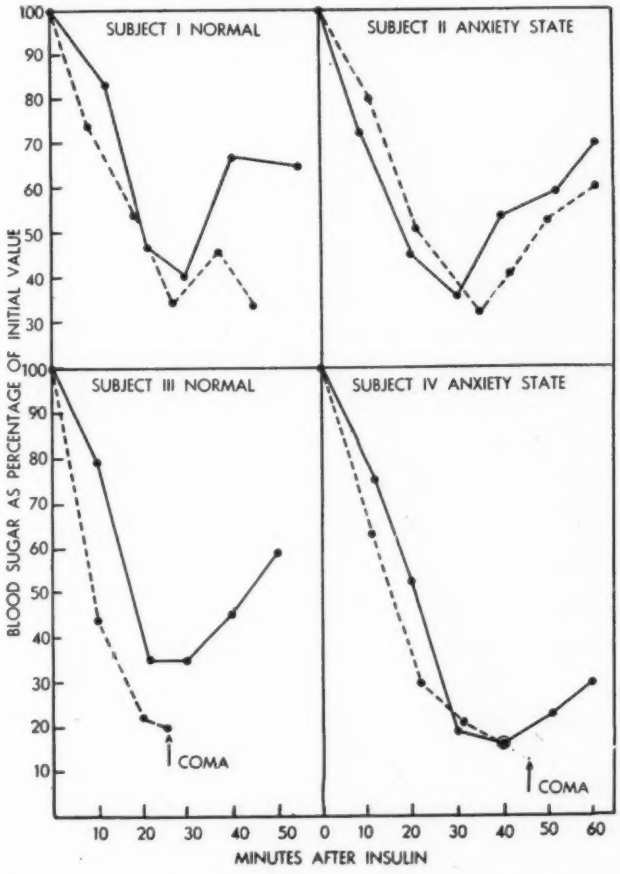

FIG. 2.—Blood sugar curves from four fasting human subjects. ●———● insulin alone (intravenously). ●- - -● same dose of insulin given 10 min. after 100 mg. hexamethonium iodide intramuscularly. Subjects I–III: 0.1 i.u. insulin/kg.; subject IV: 0.26 i.u. insulin/kg. (No blood specimen was obtained during coma from subject IV.)

Man.—Curves constructed from data obtained from four human subjects are shown in Fig. 2. Here also hexamethonium caused a more depressed blood sugar curve, though the results were less striking, probably because the dose of hexamethonium was less (1.4 mg./kg.). Nevertheless, two of the subjects became comatose when hexamethonium was given, although they did not lose consciousness with the same dose of insulin alone. Consciousness was quickly restored by intravenous glucose.

Symptoms in man

Sweating was marked in all cases when insulin was given alone, in some enough to necessitate a change of clothing. When hexamethonium was given in addition there was no sweating.

Tremor of the outstretched hand was strikingly less when hexamethonium was given.

A sharp rise in the *heart rate* occurred, just before the blood sugar reached its lowest point, when insulin was given alone. With hexamethonium the heart rate increased steadily from the beginning of the experiment and remained high without a sharp peak.

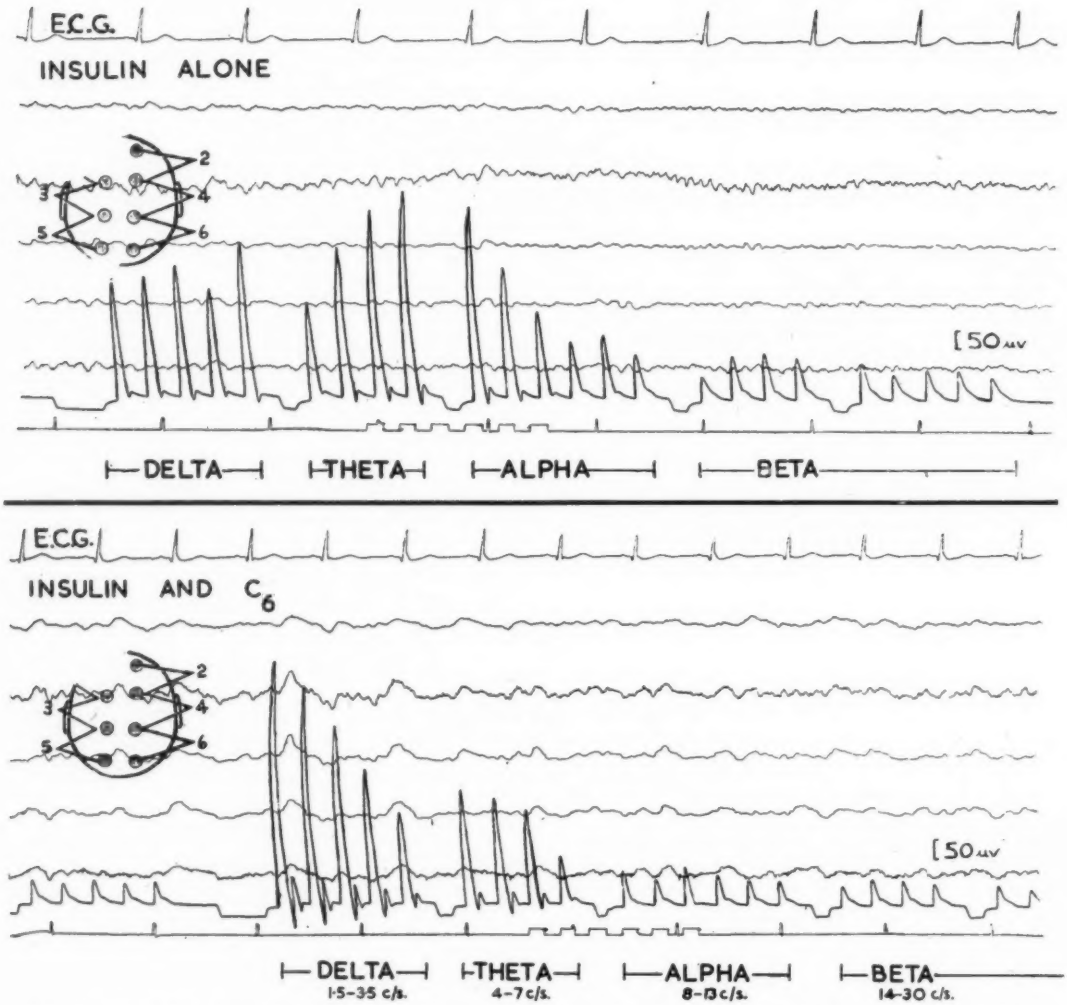

FIG. 3.—E.E.G.s from Subject IV. Above: after insulin alone (20 i.u. intravenously). Below: after the same dose of insulin with hexamethonium iodide (100 mg. intramuscularly). The blood sugar at the time these records were taken was the same, namely, 15 mg./100 ml. The bands of the analyser epoch are indicated under each tracing. Signal: 1 sec.

Hexamethonium caused a striking modification in the *subjective effects* of insulin hypoglycaemia. Restlessness and anxiety were markedly diminished in all cases, although the hypoglycaemia was greater, and two subjects passed peacefully through a stage of drowsiness to coma. One of our subjects (Subject IV) had previously received insulin treatment for his mental condition and had needed manual restraint. With hexamethonium no excitement occurred and he passed into unconsciousness without comment or movement. Subjects noticed palpitations less when hexamethonium had been given.

The *E.E.G. records* were similar whether hexamethonium had been given or not. As the blood sugar fell, *theta* activity appeared, became dominant, and was succeeded by the development and sometimes dominance of *delta* activity. *Alpha* activity diminished and sometimes disappeared. A comparison of tracings from the same subject at the same blood sugar levels showed a greater shift towards slower activity on the occasions when hexamethonium was given (Fig. 3).

Effect of Hexamethonium on the Convulsant Blood Sugar Level

Groups of 20 male mice of uniform weight were used. They had been fed on bread and milk for three days and starved for twenty-four hours. All received a subcutaneous injection of insulin and half received hexamethonium (5 mg./kg.) intraperitoneally simultaneously. They were then all placed in an incubator at 33° C. As each mouse developed convulsions a specimen of blood was taken either from the tail (Exp. 1) or by decapitation (Exps. 2 and 3) and the blood sugar estimated by King's method (1947). The results are recorded in Table II. There was no significant difference between the mean blood sugar at the development of hypoglycaemic convulsions in the two groups.

TABLE II

Effect of hexamethonium iodide (5 mg./kg. i.p.) on the mean blood sugar level at which insulin convulsions develop (mice)

Exp.	No. of mice in each group	Dose of insulin i.u./mouse	Mean blood sugar on convulsion, mg./100 ml. blood	
			Without hexamethonium	With hexamethonium
1	10	0.2 s.c.	17 ± 2	15 ± 4
2	10	0.2 s.c.	9 ± 3	10 ± 3
3	8	0.4 s.c.	14 ± 4	11 ± 6

Discussion

The experiments described indicate that hexamethonium potentiates the convulsant action of insulin by increasing the degree of hypoglycaemia. This is due to blocking of the hypoglycaemia-induced sympathetic discharge to the liver and adrenal medulla. Cannon *et al.* (1924) obtained a similar result by adrenalectomy and section of the hepatic nerves.

The other changes in the response to hypoglycaemia can also be attributed to the same action. The absence of sweating, of increased tachycardia, and of palpitations was to be expected as these symptoms occur with sympathetic stimulation.

Tremor of the hands, restlessness, and anxiety occur during intravenous infusion of adrenaline in man, and these symptoms, present when insulin was give alone, were absent when hexamethonium was given with it. Adrenaline infusion in man favours the development of fast rhythms in the E.E.G. (Gibbs and Maltby, 1943). Studies on the patients in this series showed that at the same low blood sugar levels slower rhythms were more marked with hexamethonium and insulin than with insulin alone. This may be due to the absence of adrenaline from the circulation in the former case.

Feldberg (1945) suggested that hypoglycaemic convulsions might be due to an accumulation of acetylcholine in the brain through the removal of the "glucose brake" on acetylcholine synthesis. We have shown that hexamethonium antagonizes nicotine convulsions possibly by a central action; it was of interest to see whether hexamethonium antagonized hypoglycaemic convulsions. The experiments described in the last section failed to demonstrate any such action.

A clinical application for some of these results can be suggested. A diabetic receiving a ganglionic blocking agent in the course of treatment for hypertension or any other condition would be unlikely to have any of the warning symptoms of a hypoglycaemic reaction and might slip quietly into coma as did two of the subjects in this series.

The relation of this work to the smooth induction of insulin coma in psychiatric therapy might be worthy of further investigation.

While this paper was in preparation a paper by Schachter (1951) appeared in which he showed that hexamethonium causes an increased sensitivity to insulin in unanaesthetized dogs.

Summary

1. The effect of hexamethonium on insulin hypoglycaemia in man and in rabbits has been examined.

2. Hexamethonium (i) potentiates the convulsant action of insulin by increasing the degree of hypoglycaemia, (ii) abolishes or markedly alters the symptoms of insulin hypoglycaemia in man, and (iii) modifies the E.E.G. changes in hypoglycaemia.

3. The relation of these effects to sympathetic blockade is discussed.

4. The blood sugar level at which insulin convulsions develop is not affected by hexamethonium.

5. Attention is drawn to the possibility of symptomless hypoglycaemia occurring when insulin and hexamethonium are used together.

It is a pleasure to thank Dr. W. Sargant for clinical facilities, Dr. J. T. Hutchinson for interpreting the E.E.G.s, and Miss Slater and Mr. Cowland for technical assistance.

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THE INHIBITORY ACTION OF TRI-*ORTHO*-CRESYL PHOSPHATE ON CHOLINESTERASES

BY

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The toxic effects of tri-*o*-cresyl phosphate (TOCP) in man have been observed since the end of the last century. Lorot (1899) described six cases of multiple neuritis in patients with pulmonary tuberculosis treated with preparations of "phosphocreosote" which were subsequently found to contain TOCP. Further outbreaks of paralysis associated with poisoning by this compound have since been reported. The TOCP ingested in these cases was found to have been present as an adulterant in drinks prepared from extracts of Jamaica ginger (Smith and Elvove, 1930a, b), in apiol (Ter Braak, 1931 ; Germon, 1932), and in edible oils (Sampson, 1938 ; Hotston, 1946). TOCP is now used in the plastics industry and cases of poisoning among workers in this country have been described by Hunter, Perry, and Evans (1944).

The way in which this compound produces these paralytic symptoms is at present unknown. Smith and Lillie (1931) have described the histopathology of the condition in man and also the lesions produced by TOCP in the peripheral nerves and the central nervous system of experimental animals. They found evidence of demyelination of the peripheral nerves, degenerative changes in the anterior horn cells, and "fatty degeneration" in the white substance of the spinal cord.

On the assumption that the motor paralysis induced by TOCP might be associated with a lesion of the motor endplates in the affected muscles, Bloch in 1941 began a study of the action of this compound on cholinesterase activity. He first showed that the cholinesterase of horse serum was inhibited by low concentrations of this compound. Two years later Hottinger and Bloch (1943) demonstrated that the hydrolysis of acetylcholine by human and rabbit serum, liver, and brain was also inhibited by TOCP ; in addition, the splitting of tributyrin by serum and liver was found to be diminished by this compound, although serum phosphatase and pancreatic lipase were unaffected. No attempt was made at that time to study the different types of cholinesterase by means of selective substrates.

In a brief report it was next stated (Mendel and Rudney, 1944) that the oral administration of TOCP to rats caused a lowering of the serum pseudo-cholinesterase, whereas the true cholinesterase appeared to be unaffected ; Myers and Mendel (1949) later claimed that the hydrolysis of tributyrin by rat brain was insensitive to TOCP.

The experiments described below were therefore carried out to investigate this inhibition of cholinesterases in greater detail.

It seemed desirable in the first instance to compare the sensitivities to TOCP of "true" and "pseudo" cholinesterases from different sources. As substrate for the true cholinesterase present, for example, in human erythrocytes and in the grey matter of the central nervous system, we have used acetyl-β-methylcholine (Mendel, Mundell, and Rudney, 1943); for the pseudo-cholinesterase we have in most cases used butyrylcholine (Stedman, Stedman, and Easson, 1932), since this compound is hydrolysed much more rapidly than benzoylcholine by human serum cholinesterase, and since Adams (1949) has reported that it is hydrolysed by the true cholinesterase of human erythrocytes at only 5 per cent of the rate at which acetyl-β-methylcholine is hydrolysed. From the symptomatology of TOCP poisoning the esterases of the central nervous system and of the motor endplates seemed specially worthy of study, and, in view of the finding by Ord and Thompson (1951) of a relatively high level of pseudo-cholinesterase in the white matter of the nervous system, samples of grey and white matter from the human cerebrum were among the tissues employed. In addition to human material, tissues were taken from the chicken, rabbit, and rat. The chicken and rabbit were chosen, since previous workers had succeeded in producing paralysis with TOCP in these animals, whereas the rat had been found insensitive (Smith, Engel, and Stohlman, 1932; Sampson, 1938).

Methods

Estimation of esterase activity was carried out manometrically at 38° and at *p*H 7.4, the Warburg apparatus being used. All measurements were done in duplicate, and corrections were made for non-enzymic hydrolysis of the substrate. Esterase activity of serum is expressed as μl. CO_2/ml./min.

Tissue preparations

Human serum or oxalated plasma was diluted with 3 vol. of 0.025 M-$NaHCO_3$; 0.2 ml. of this dilution was added to each flask.

Human erythrocytes were obtained from oxalated blood; the cells were centrifuged, washed twice with 0.9 per cent (w/v) NaCl, and lysed with a volume of glass-distilled H_2O equal to twice the volume of the original sample of blood; 0.2 ml. of this haemolysate was added to each flask.

Human brain, spinal cord, sciatic nerve, and muscle were obtained fresh from autopsies. The grey matter from the cerebral cortex or white matter from the subcortical tracts was dissected out, washed in 0.9 per cent NaCl, and homogenized in 0.025 M-$NaHCO_3$. A segment of spinal cord was dissected free from meninges and treated similarly. Since it was found difficult to prepare a satisfactory homogenate of sciatic nerve on account of the fibrous elements present in this tissue, a weighed portion of the nerve was ground with washed sand and 0.025 M-$NaHCO_3$, centrifuged, and re-ground twice; the supernatant after each centrifugation was pipetted off and the volume made up to give a known dilution of the original weight of the nerve. The intrinsic muscles of the tongue and the adductor pollicis were chosen as sources of motor endplate cholinesterase, since these muscles, subserving fine movements, are richly innervated and might therefore be expected to show a high overall content of true cholinesterase resulting from a relatively high concentration of motor endplates within the muscle.

Nerve tissues from other animals were prepared in the same way, the animals being killed by air-embolism or decapitation.

Chicken and rat serum were obtained from blood collected by decapitation; 0.2 ml. of undiluted serum was added to each flask.

Substrates

(1) Acetylcholine chloride (ACh) (British Drug Houses, Ltd.);

(2) Butyrylcholine chloride (BuCh) (British Drug Houses, Ltd.);

(3) Acetyl-β-methylcholine chloride (MCh) (Savory & Moore, Ltd.);

(4) Benzoylcholine chloride (BCh) prepared by Dr. A. H. Ford-Moore, Experimental Station, Porton;

(5) Tributyrin (TB) (British Drug Houses, Ltd.).

The choline esters were dissolved in 0.025 M-$NaHCO_3$ immediately before use to give a final concentration of 0.015 M for ACh and BCh and 0.03 M for BuCh and MCh. The tributyrin was pipetted directly into the side-arm of the Warburg flask (0.03 ml. per flask). The zero reading was always taken 3 min. after tipping in the substrate from the side-arm.

Inhibitors

(1) Tri-*o*-cresyl phosphate (TOCP) prepared from pure *o*-cresol (Geigy Pharmaceutical Laboratories, Ltd.);

(2) Di-*iso*propyl fluorophosphonate (DFP), kindly provided by the Experimental Station, Porton.

Owing to the low solubility of TOCP in water, only the lowest concentrations that we have used have given optically clear solutions. With the higher concentrations, in most experiments, a fine, stable suspension of TOCP in 0.025 M-$NaHCO_3$ was used although in some of our earlier experiments it was dissolved in *iso*propanol before adding to the flasks. Since *iso*propanol has a potentiating effect on cholinesterase activity (Todrick, Fellowes, and Rutland, 1951), an equal volume of *iso*propanol (0.05 ml.) was added to the control flask in these experiments. A given concentration of TOCP, however, was found to produce roughly the same degree of inhibition whether dissolved in *iso*propanol or suspended in $NaHCO_3$.

Since, with the higher concentrations of TOCP, we have not been using a true solution of our inhibitor we have expressed our results in terms of the total amount of TOCP added to each Warburg flask; assuming complete solution, 100 μg. per flask (3 ml.) = 0.91×10^{-4}M.

The DFP was dissolved in 0.025 M-$NaHCO_3$ immediately before use. In all experiments 25–35 min. elapsed between the addition of the inhibitor to the enzyme preparation and the time at which the substrate was tipped in.

RESULTS

Human tissues

The effects of different concentrations of TOCP on the esterase activities of a number of different human tissues are shown in Table I. All the figures given for percentage inhibitions in this Table are the means of at least two experiments. It will be seen that a very striking difference exists between the sensitivities to this compound of the true and pseudo-cholinesterases, when MCh and BuCh respectively are used as substrates. The pseudo-cholinesterase activities of serum, white and grey matter from the cerebrum, and spinal cord were each inhibited approximately 50 per cent by 50 μg. TOCP per 3 ml. reaction mixture; sciatic nerve appears to be somewhat more sensitive, a mean inhibition of 59 per cent being caused by 25 μg. TOCP per 3 ml., i.e., approximately 2×10^{-5}M. Using a purified

TABLE I

INHIBITION BY TOCP OF HUMAN CHOLINESTERASES AND TRIBUTYRINASE

Enzyme and source	Percentage inhibition of human esterases by TOCP at								
	25	50	100	150	200	300	500	1,000	2,000
	μg./3 ml.								
Pseudo-cholinesterase (substrate 0.03 M-BuCh)									
Serum	27	55	73	82	93	—	—	—	—
White matter (cerebrum)	38	65	81	87	91	—	—	—	—
Grey matter (cerebrum)	33	51	66	72	86	—	—	—	—
Spinal cord	39	55	64	64	74	—	—	—	—
Sciatic nerve	59	81	92	97	99	—	—	—	—
True cholinesterase (substrate 0.03 M-MCh)									
Erythrocytes	—	7	4	1	7	10	4	—	—
White matter	—	—	—	9	9	—	—	—	—
Grey matter	5	4	9	6	8	—	—	—	—
Spinal cord	—	—	2	8	7	6	2	13	22
Striated muscle	—	3	8	—	10	15	11	31	—
Tributyrinase (substrate 0.03 ml. TB/3 ml.)									
White matter	—	21	21	—	21	—	31	—	—
Grey matter	—	—	—	—	—	—	28	30	40
Spinal cord	—	21	30	—	33	—	—	—	—

human plasma cholinesterase (Fraction IV-6) Brauer (1948) reported 50 per cent inhibition of activity by approximately 10^{-7}M-TOCP. Experiments with BCh as substrate for the pseudo-cholinesterase of serum, cerebrum (white matter), and sciatic nerve gave inhibitions of the same order as those obtained in the presence of BuCh.

The true cholinesterase in erythrocytes, white and grey cerebral matter, spinal cord, and striated muscle, on the other hand, was not significantly inhibited by concentrations of this order, and only 2–25% inhibition was produced by concentrations as high as 500–2,000 μg. per 3 ml. Tributyrinase activity, as reported earlier by Hottinger and Bloch (1943), is also inhibited by TOCP, although to a very much smaller extent than the pseudo-cholinesterase, concentrations of TOCP as high as 2,000 μg. per 3 ml. producing only up to 40 per cent inhibition.

Animal tissues

Similar *in vitro* experiments have been carried out with tissues from rabbits, chickens, and rats as a preliminary to extending these observations to a study of *in vivo* intoxication in laboratory animals. The results are summarized in Table II.

With rabbit brain and spinal cord there is also preferential inhibition of the pseudo-cholinesterase as indicated by BuCh hydrolysis. Thus, 200 μg. TOCP per 3 ml. inhibits BuCh hydrolysis 50 per cent and MCh hydrolysis only 10–18 per cent. The true cholinesterase in skeletal muscle is also insensitive to inhibition by these concentrations of TOCP. Comparison of these results with those given in Table I will show that the pseudo-cholinesterase in the rabbit central nervous system is somewhat less sensitive to inhibition by TOCP than the corresponding enzyme in

TABLE II

EFFECT OF TOCP ON ANIMAL CHOLINESTERASES

Enzyme and source	Percentage inhibition of animal esterases by TOCP at									
	50	100	200	400	500	800	1,000	1,200	1,500	2,000
	μg./ 3 ml.									
Pseudo-cholinesterase (substrate 0.03 M-BuCh)										
Rabbit Brain	—	36	49	62	—	75	81	—	82	82
,, Spinal cord ..	—	37	52	72	—	80	86	—	89	—
Chicken Serum	39	60	62	94	—	—	—	—	—	—
,, Brain	—	—	33	48	57	68	72	—	—	—
,, Spinal cord ..	8	19	39	50	60	88	94	—	—	—
,, Nerve	—	26	28	38	46	—	—	—	—	—
Rat Brain	—	—	9	27	—	39	41	51	54	62
,, Serum	—	5	8	—	8	14	15	—	—	—
True cholinesterase (substrate 0.03 M-MCh)										
Rabbit Brain	—	6	18	12	—	17	9	—	15	—
,, Spinal cord ..	—	4	10	16	—	16	14	—	15	—
,, Muscle	—	—	0	3	—	—	3	—	9	22
Chicken Brain	—	—	1	—	10	—	12	—	24	—
,, Spinal cord ..	—	—	18	—	25	—	31	—	41	—
Rat Brain	—	7	—	0	—	0	8	—	—	—
,, Spinal cord ..	—	—	—	—	0	—	10	—	—	4
Tributyrinase (substrate 0.03 ml. TB/3 ml.)										
Rabbit Brain	—	—	24	32	—	35	47	—	51	43

human tissues ; this agrees with the earlier finding by Hottinger and Bloch (1943) concerning overall hydrolysis of acetylcholine in the tissues of these two species. Tributyrinase activity of rabbit brain is only partially inhibited by high concentrations of TOCP.

Hydrolysis of BuCh by chicken serum, brain, spinal cord, and peripheral nerve was also markedly inhibited by concentrations of 100–500 μg. TOCP per 3 ml., although with this species also these tissues were rather less sensitive to TOCP than the corresponding human ones. It was found, however, that chicken serum hydrolyses both BuCh and MCh. Summation experiments with these two substrates gave no evidence of any increased rate of hydrolysis over that observed with BuCh alone ; thus in two experiments the rates in μl. CO_2 per ml. per min. were : with MCh alone 9.4 and 8.3 ; with BuCh alone 21.6 and 21.0 ; and with MCh plus BuCh 20.6 and 17.8. This failure to observe summation in the presence of both substrates might be due to inhibition of the MCh hydrolysis by the BuCh, as indeed has been found to be true with MCh and BCh hydrolysis by rat serum (unpublished observations by Ord and Thompson) or to the hydrolysis of both substrates by a single enzyme. If, however, two distinct enzymes were concerned with the hydrolysis of these two substrates it might be expected that, as with the hydrolysis of BuCh and MCh by the tissues of other species, they would show different degrees of sensitivity to inhibition by DFP. Experiments were therefore carried out to compare the inhibition of hydrolysis of these two substrates by both DFP and TOCP. It was found that with both these inhibitors, which in all other species so far studied have

proved themselves to be selective in their actions on the true and pseudo-cholinesterases, almost identical degrees of inhibition of the hydrolysis of BuCh and MCh were obtained ; thus with 3×10^{-9}M-DFP the percentage inhibitions were, for BuCh, 79 and, for MCh, 78 ; similarly with 200 μg. TOCP per 3 ml. the inhibitions were, for BuCh, 55 and, for MCh, 58 per cent. This suggests that these two substrates are hydrolysed by the same enzyme. Augustinsson (1948) has also reported on the relatively high rate of hydrolysis of MCh by fowl plasma, although he did not carry out summation or inhibition experiments to characterize the enzyme concerned. It would seem therefore that chicken serum contains a cholinesterase which does not conform to either the " true " or " pseudo " type, as judged by its action on selective substrates, inasmuch as it appears to hydrolyse both BuCh and MCh. However, since it hydrolyses BuCh more rapidly than MCh, and since it is DFP-sensitive, it is proposed for present purposes to regard it as a pseudo-cholinesterase.

By analogy with human tissues, in which the pseudo-cholinesterase in the brain and in the serum are similar in respect of substrate specificity, it might be expected that the pseudo-cholinesterase in chicken brain would also hydrolyse both BuCh and MCh, and that MCh hydrolysis by this tissue would therefore also be sensitive to TOCP. It will be seen from Table II, however, that MCh hydrolysis by chicken brain is hardly affected by concentrations of TOCP that cause over 50 per cent inhibition of BuCh hydrolysis.

The presence of a true cholinesterase in chicken brain, which like that in human and rabbit brain hydrolyses MCh and is insensitive to TOCP, would explain this failure of TOCP to inhibit MCh hydrolysis, particularly if, as in other species, it is present in much greater amount than the pseudo-cholinesterase. A comparison of the effects of DFP on the hydrolysis of these two substrates by chicken brain shows that a true cholinesterase is present in addition to the enzyme hydrolysing BuCh ; thus, BuCh hydrolysis by chicken brain is inhibited 72 per cent by 3×10^{-9}M-DFP, but MCh only 2 per cent.

In this connexion it is interesting to note (Table II) that although MCh hydrolysis by chicken brain, a tissue containing a large preponderance of the true cholinesterase, is only very slightly inhibited by TOCP, MCh hydrolysis by the spinal cord is significantly more sensitive ; this increased sensitivity to inhibition by TOCP may be explained by differences in the relative amounts of true and pseudo-cholinesterase in the brain and spinal cord. Thus BuCh is hydrolysed at 65–80 per cent of the rate of MCh by chicken spinal cord, whereas the corresponding figure for chicken brain is only 15–20 per cent ; in chicken spinal cord therefore the TOCP-sensitive pseudo-cholinesterase will contribute very appreciably to the hydrolysis of MCh.

We can therefore conclude that in the central nervous system of the chicken, as in other species, two cholinesterases are present, one hydrolysing BuCh and MCh and sensitive to TOCP and DFP, and the other relatively insensitive to these inhibitors, active against MCh, and therefore presumably a true cholinesterase.

Lastly a few experiments have been carried out with tissues from albino rats. In contrast to our findings with human, rabbit, and chicken tissues, the pseudo-cholinesterase of rat brain and serum, as indicated by BuCh hydrolysis, is not very sensitive to TOCP, and in no experiment did we obtain more than 62 per cent inhibition.

Discussion

Although TOCP has been described as an inhibitor of cholinesterase (Bloch, 1941), the experiments reported above indicate that in the species studied by us the true cholinesterase of nerve tissue, striated muscle, and erythrocytes is not inhibited to any major degree by amounts of TOCP even far in excess of its solubility. On the other hand, the pseudo-cholinesterases of human, rabbit, and chicken serum and nerve tissues are inhibited by relatively low concentrations of this compound *in vitro* ; of these three species the enzyme in human tissues is rather more sensitive to TOCP than that in the rabbit and chicken. The pseudo-cholinesterase of the rat nervous system is, however, very much less sensitive. These species variations of *in vitro* sensitivity of pseudo-cholinesterase to inhibition by TOCP are of interest when compared with the differing sensitivities of these species to *in vivo* intoxication by TOCP ; thus, whereas flaccid paralysis and death can be readily brought about in the rabbit and the chicken by orally or parenterally administered TOCP, the albino rat does not appear to be susceptible to this poison (Smith, Engel, and Stohlman, 1932 ; Mendel and Rudney, 1944). De Vaal (1948) has stated that 1 ml. of TOCP given orally to " rats of our own breed " causes motor disturbances (convulsions, spastic paralysis) and death after 14–16 days. We have, however, administered 0.5 ml. subcutaneously to albino rats weighing 150–200 g. without adverse effect.

The selective inhibition of pseudo-cholinesterase by TOCP *in vitro* raises the question of the possible significance of these findings in connexion with the toxicology of this compound. Koelle and Gilman (1949) have recently revived the earlier suggestion that the flaccid paralysis produced in man by this compound might be due to prolonged inhibition of the cholinesterase at the motor endplates with a resulting neuromuscular block. Our finding that the true cholinesterase in skeletal muscle is not significantly inhibited by concentrations of TOCP which cause almost complete inhibition of the pseudo-cholinesterase does not support this view. Further, if this were the explanation it is not easy to understand why a period of approximately 10–20 days normally elapses before the onset of the paralysis. It must be remembered also that, with the possible exception of some symptoms of gastro-intestinal upset shortly after the oral administration of this compound, no typical signs or symptoms of cholinergic over-activity are produced by TOCP, whereas these are usually striking after intoxication with DFP or the organo-phosphorus insecticides.

The essential histological change in the motor neuritis brought about by TOCP is a degeneration of the myelin sheaths of the peripheral nerves together with a fatty degeneration of the white matter of the cord, and Smith and Lillie (1931) have described this compound as a " specific myelin poison." It might therefore be expected that from the biochemical point of view also the toxicological action of TOCP might be centred round the myelin-containing regions of the nervous system, and Ord and Thompson (1952) have previously shown that the pseudo-cholinesterase of the central nervous system appears to be associated, particularly with the white fibre tracts. The possibility must therefore be considered that the pseudo-cholinesterase of nerve tissue may be connected in some way with the maintenance of the myelin sheaths of nerve fibres, and that inhibition of this enzyme by TOCP may play a

part in the production of the demyelination and consequent paralysis. Further *in vivo* experiments are now in progress in the hope of testing this hypothesis.

Since pseudo-cholinesterase is widely distributed in different tissues throughout the body (Ord and Thompson, 1950), it might be expected that widespread evidence of tissue dysfunction, though not necessarily with cholinergic symptoms, would be present in TOCP intoxication if the underlying mechanism of its toxic action was simply an inhibition of this enzyme. It must be pointed out, however, that TOCP differs very markedly from eserine, DFP, and most of the organo-phosphorus insecticides in its solubility properties ; it is only very slightly soluble in water, but readily soluble in lipoid solvents. Its distribution in the body and its access to the active centre of the tissue pseudo-cholinesterases may therefore be influenced by this, and the fatty nature of the white matter of the nervous system may facilitate its uptake by this tissue.

The insolubility of TOCP in water has indeed presented some practical problems in the course of this work. Preliminary observations have shown that TOCP is soluble in 0.025 M-$NaHCO_3$ to the extent of about 8 μg. per ml., as judged by optical clarity of the resulting solution. In the Warburg flasks containing 25 μg. per 3 ml. the TOCP may therefore be expected to be all in true solution ; with the higher concentrations of TOCP which we have used, the compound was added as a suspension. However, in view of the fact that we do not know what fraction of the amount added ultimately dissolves in the fat of the tissue preparation present as our enzyme source, it is not possible to form any quantitative opinion as to the partition of the added TOCP between the different phases of the heterogeneous systems with which we have been working. The fact that Bloch (1943) demonstrated that the relatively non-toxic tri-*m* and tri-*p*-cresyl phosphates, whose solubility properties closely resemble those of TOCP, were also very much less powerful cholinesterase inhibitors suggests strongly that the inhibition which we have observed is not due merely to some physical effect of the suspended TOCP. In order to obtain further evidence that we were not dealing with a non-specific physical process we have studied the effect of the addition of the non-ionic surface-active substance Lubrol W (a cetyl alchohol-polyoxyethylene condensate) on the inhibitory action of TOCP ; the Lubrol caused a marked clearing of the emulsion, but was without effect on the degree of inhibition produced.

Finally, we have made a few preliminary observations on the degree of reversibility of the inhibition of pseudo-cholinesterase by TOCP, and have shown that dialysis for sixteen hours causes no diminution in the degree of inhibition.

Summary

1. A study has been made of the action of tri-*ortho*-cresyl phosphate (TOCP) on the true and pseudo-cholinesterases of a number of tissues of man, the rabbit, the chicken, and the rat.

2. With human tissues TOCP was found to be a selective inhibitor of the pseudo-cholinesterase, concentrations which caused 75–99 per cent inhibition of this enzyme in cerebrum, spinal cord, sciatic nerve, and serum causing only 7–10 per cent inhibition of the true cholinesterase in cerebrum, spinal cord, striated muscle, and erythrocytes.

3. With rabbit and chicken tissues a selective inhibition of the pseudo-cholinesterase was also observed, although tissues from these species were rather less sensitive to TOCP than those from man.

4. The pseudo-cholinesterase of the albino rat, an animal apparently insensitive to poisoning by TOCP, was only partially inhibited even by very high concentrations ; the true cholinesterase in this species was also insensitive.

5. The possible significance of these results is discussed in connexion with the demyelination and motor paralysis which characterize TOCP poisoning.

6. The cholinesterase of chicken plasma differs from those of other species studied in that it hydrolyses both butyrylcholine and acetyl-β-methylcholine.

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ACCUMULATION OF ACETYLCHOLINE IN BRAIN AND BLOOD OF ANIMALS POISONED WITH CHOLINESTERASE INHIBITORS

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The extreme toxicity of a group of phosphorus compounds, of which tetraethylpyrophosphate (TEPP) and di*iso*propylfluorophosphonate (DFP) are examples, is attributed to their powerful inhibition of cholinesterase. Substances of this type cause generalized cholinergic stimulation, convulsions, and neuromuscular paralysis, effects which are thought to be due to accumulation of acetylcholine at certain nerve endings throughout the body. The object of the experiments reported here was to discover whether abnormal amounts of acetylcholine were present in animals poisoned with such compounds.

METHODS

Acetylcholine was determined by means of the heart of *Venus mercenaria*, isolated according to the procedure of Tower and McEachern (1948). The isolated heart was mounted in an organ bath containing 10 ml. of Venus saline of the following composition: 30 g. NaCl, 5.1 g. $MgSO_4.7H_2O$, 0.9 g. KCl, 0.14 g. $NaH_2PO_4.H_2O$, 0.95 g. $CaCl_2$, 0.25 g. glucose, and 0.0001 g. phenol red per litre, together with sufficient 0.1 M-Na_2HPO_4 to adjust the *p*H to 7.2. The solution was stirred and oxygenated by a slow stream of air bubbles.

The spontaneous contractions of the isolated Venus ventricle were recorded with a 10-inch counterweighted gimbal lever, at tenfold magnification with a load of 0.6 g., on smoked paper moving at the rate of 0.12 mm. per sec.

Doses of standard acetylcholine bromide solution and of the solution to be assayed were added to the bath alternately at four-minute intervals, allowed to act for 70 sec., and then removed by washing out the organ bath with three changes of fresh saline. Responses were obtained for two dosage levels of both standard and unknown, twice repeated, according to a conventional two-dose assay plan, described by Bliss and Marks (1939). All timing and washing operations were performed automatically by an electric cycle timer.

The depressions of the amplitude of the recorded heartbeat were measured and expressed as a percentage of the amplitude of the normal beat (taken as a mean of the amplitude of the beat before the dose and after recovery from it). The percentage

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responses were transformed to probits, and the calculation of the concentration ratio between standard and unknown completed by the method of Bliss and Marks (1939).

In preliminary experiments rat brain was ground with sand, with addition of 0.3 mg. TEPP in 0.1 ml. propylene glycol, 1.5 ml. 0.33 N-hydrochloric acid, and 1.5 ml. Venus saline. The homogenate was transferred to a centrifuge tube, allowed to stand for 30 minutes or one hour, centrifuged, and the supernatant solution decanted and neutralized for assay. The tissue residue was mixed with additional quantities of TEPP, acid, and Venus saline as before, and allowed to stand for a second extraction period. The second supernatant solution was also neutralized and assayed. In practice it was found that at least three such extractions were necessary : thus in one experiment two extractions of 1 hr. each gave 0.74 + 0.34 = 1.08 μg. acetylcholine per g. brain ; in a second experiment three extractions of 30 min. each gave 1.08 (first two extractions) + 0.16 = 1.24 μg.

When the tube containing the tissue homogenate was shaken continuously, a considerable speeding up of the extraction resulted. The results of such an experiment, comparing opposite halves of the same rat brain, extracted with and without shaking, were as follows : Amounts of acetylcholine in μg. per g. brain obtained in four extractions of 30 min. each : (i) with shaking : 2.56 + 0.84 +0.50 + 0.14 = 4.04 ; (ii) without shaking : 1.49 + 0.99 + 0.96 + 0.47 = 3.91 ; a fifth extraction gave amounts too small for accurate measurement.

When the homogenate was placed in a boiling water bath for five minutes, for the first extraction only, and then shaken continuously for 30-minute periods as before, the extraction was slightly faster. It was decided to include this step in the procedure in order to prevent any enzymic accumulation or destruction of acetylcholine in the extracts. Three extractions with continuous shaking were considered to be enough, because a fourth would contain less than 10 per cent of the acetylcholine already extracted, an increment which approximates the limits of accuracy of the biossay for acetylcholine.

The procedure finally adopted for making brain extracts was as follows : the rat was killed (by the particular method selected), and the whole brain dissected out, including the cerebellum and a variable proportion of the medulla oblongata. The tissue was blotted free of blood, weighed, and transferred to a mortar with 0.3 mg. TEPP in 0.1 ml. propylene glycol, 1.5 ml. 0.33 N-hydrochloric acid, 1.5 ml. Venus saline, and a sufficient quantity of washed sand. The brain was rapidly and thoroughly ground to a paste. The homogenate was transferred to a centrifuge tube with the addition of three 0.5 ml. portions of Venus saline. The tube was immersed in a boiling water bath for five minutes, with occasional shaking. The tube was cooled, stoppered, and shaken by machine, in a longitudinal direction for thirty minutes. The tube was centrifuged, and the supernatant fluid decanted ; 1.5 ml. 0.33 N-hydrochloric acid and 1.5 ml. Venus saline were added to the tissue residue, and shaking repeated for a second 30-minute period. After centrifugation, the supernatant was removed, and the tissue residue was extracted for a third time. The combined extract was brought to *p*H 6.8 by cautious addition of 0.33 N-sodium hydroxide solution in the presence of a drop of 0.1 per cent phenol red. The extract was slightly reacidified with one drop of 0.33 N-hydrochloric acid, and stored in the frozen state. For assay, the solution was thawed out and diluted to 25 ml. with Venus saline.

In the first experiment, rats were killed in groups of four, one by each of the four methods : (1) asphyxia in nitrogen gas containing 5 per cent carbon dioxide ; (2) intraperitoneal injection of 1 mg. strychnine sulphate per kg. ; (3) chloroform inhalation ; (4) intraperitoneal injection of 2 mg. TEPP per kg. The order of killing was systematically rotated according to a Latin square design in order to avoid any bias due to the order of killing.

Brains were extracted and assayed on the day on which the rats were killed, a single Venus mercenaria heart preparation being used. The whole procedure was repeated on four different days, thus completing a 4 × 4 Latin square.

In the second experiment the same methods of killing and preparation of brain extracts were used, but the extracts were stored until all the rats had been killed ; assays were then done on groups of four extracts, the groups taken vertically through the Latin square, so that four extracts assayed together represented the four methods of killing but taken on successive groups of rats.

In the third experiment control rats were killed by asphyxia, as before. The three other rats in each group were killed by intraperitoneal injection of 8.0 mg. DFP per kg. ; 4 mg. Paraoxone (diethyl *p*-nitrophenyl phosphate) per kg. ; or 3 mg. eserine sulphate per kg. The Latin square design was completed in the same way as the second experiment, and the extracts were stored an average of six days. Eight extra animals were included to provide additional data.

RESULTS

Comparison of the effect of TEPP poisoning on the acetylcholine content of rat brain, with those of asphyxia, chloroform anaesthesia, and strychnine poisoning

The object of this experiment was to find whether TEPP poisoning caused an increase in the acetylcholine content of rat brain, as might be expected on theoretical grounds. To do this necessitated a comparison of brains from TEPP poisoned animals and from " normal " control animals. The nature of the " normal " control group posed a problem, because physiological factors are known to affect the acetylcholine content of the brain (Richter and Crossland, 1949). Certain physiological disturbances associated with TEPP poisoning could alter the acetylcholine content of the brain, without the change being associated directly with the specific anticholinesterase action of TEPP. For example, convulsions occur during TEPP poisoning, and Elliott, Swank, and Henderson (1950) have shown that convulsants decrease the acetylcholine content of the brain. Furthermore, anoxia, which invariably accompanies severe TEPP poisoning, is also known to lower the acetylcholine content (Welsh, 1943). It was decided to use two groups of controls, one group killed by a lethal dose of the convulsant strychnine, the other asphyxiated in an atmosphere of nitrogen containing 5 per cent of carbon dioxide ; these methods of killing would seem to approximate to the conditions to be expected during TEPP poisoning, apart from the specific anticholinesterase effect. However, in order not to load the experiment too heavily in favour of the expected result, a third group of rats were killed with chloroform. Anaesthesia increases the acetylcholine content of brain (Elliott, Swank, and Henderson, 1950), and consequently it was thought that, if the acetylcholine content of the brains of TEPP poisoned rats was greater than that of anaesthetized animals, the increase could be regarded as outside " normal " limits, and attributable to the specific cholinesterase inhibiting action of TEPP.

The results of these two experiments are shown in Table I. Within each assay, acetylcholine was present in greater quantity in the brain extract from the TEPP poisoned animal than from any of the controls. The differences between the means for different treatments, as tested by Fisher's " t " test applied to paired data, are all statistically significant, except that between asphyxia and strychnine.

The assays of extracts in the second experiment show considerably lower values compared to the first. The difference is attributed to destruction of acetylcholine during storage in the second experiment; the mean storage period was approximately ten days.

TABLE I

ACETYLCHOLINE FROM BRAINS OF RATS, KILLED BY ASPHYXIA, STRYCHNINE, CHLOROFORM, AND TEPP

	μg. Acetylcholine per g. brain			
	Asphyxia	Strychnine	Chloroform	TEPP
First experiment				
Assay No. 1	3.2	2.3	2.2	4.4
2	3.3	2.2	3.3	4.1
3	6.6	6.8	6.9	10.7
4	2.8	3.1	2.3	4.6
Means	3.97	3.60	3.67	5.98
Second experiment				
Assay No. 1	1.2	2.4	3.5	3.4
2	1.1	1.0	1.7	2.3
3	1.5	1.8	2.5	2.5
4	1.9	1.1	1.6	2.7
Means	1.42	1.57	2.32	2.72
Grand Means	2.69	2.58	3.00	4.35

Analysis of variance applied to the data revealed a highly significant difference between assays done with different Venus hearts. This phenomenon, which was also encountered by Welsh (1943), is difficult to understand, although it does not affect the main conclusion of the experiment. Several possible causes may be postulated, all invoking differential responses by different Venus hearts to some substance or substances in brain extracts other than freely dissolved acetylcholine.

The results of the third experiment are shown in Table II. In this experiment differences between the effects of the different cholinesterase inhibitors are relatively unimportant, because they may merely reflect differences in the arbitrary doses used. The important point is their common property of increasing the acetylcholine content of the brain above the physiological range. The differences between the results from the asphyxiated controls and each of the three groups killed with cholinesterase inhibitors were statistically significant.

Appearance of free acetylcholine in blood of animals poisoned with cholinesterase inhibitors

The previous experiments show that when rats are killed with cholinesterase inhibitors, more acetylcholine can be extracted from their brains than when they are killed by asphyxia or anaesthesia. It seems likely that, when the cholinesterase is

TABLE II

ACETYLCHOLINE FROM BRAINS OF RATS, KILLED BY ASPHYXIA, DFP, PARAOXONE, AND ESERINE

	μg. Acetylcholine per g. brain			
	Asphyxia	DFP	Paraoxone	Eserine
Assay No. 1	1.7	3.7	3.7	3.4
2	2.1	2.3	2.2	1.4
3	0.5	2.3	4.4	2.5
4	1.8	4.5	1.6	1.6
5	2.8	9.6	—	7.3
6	1.7	3.0	7.3	5.0
	Means 1.77	4.23	3.84	3.53

inhibited, acetylcholine accumulates at synapses throughout the brain, causing widespread stimulation of neurones, followed by paralysis. It should be pointed out, however, that the acetylcholine content of these extracts does not necessarily represent free acetylcholine in the brains from which the extracts were made. The acetylcholine in normal brain is probably almost entirely "bound" to brain cells in some form ready to be released upon the arrival of nerve impulses. The cholinesterase content of brain is so great that free acetylcholine must exist only very briefly and in small quantities.

If free acetylcholine does indeed accumulate in the brain and other tissues during anticholinesterase poisoning, it should appear in the blood. The following experiments demonstrate that this occurs.

Rats were given lethal doses (4 mg. per kg. intraperitoneally) of paraoxone. When signs of severe intoxication appeared, but before respiration failed, the rats were decapitated, and blood was collected. The blood was mixed with 50 units of heparin and 0.05 mg. paraoxone, to prevent clotting and loss of acetylcholine. Control rats were lightly anaesthetized with ether, decapitated, and blood collected in the same way.

The blood samples were immediately tested for the presence of acetylcholine by adding 1.0 ml. quantities of the whole blood to a 10 ml. bath containing an isolated *Venus mercenaria* heart, which had been sensitized by previous treatment with DFP.

Five pairs of rats were used. In every pair blood from the control rats either contained no detectable acetylcholine, or a very small amount, always less than 0.005 μg. per ml. blood. The blood samples from rats poisoned with paraoxone all contained definite amounts of acetylcholine, varying from 0.008 to 0.03 μg. per ml. blood. The kymograph tracing from a typical experiment is shown in Fig. 1.

In a single experiment, a rhesus monkey was anaesthetized with sodium pentobarbital. Venous blood samples taken during anaesthesia contained no detectable acetylcholine. DFP was given intraperitoneally in divided doses to a total of 4 mg. per kg. Acetylcholine was detected in a final blood sample taken just as respiration ceased, but in none of the previous samples. The kymograph record is shown in Fig. 2.

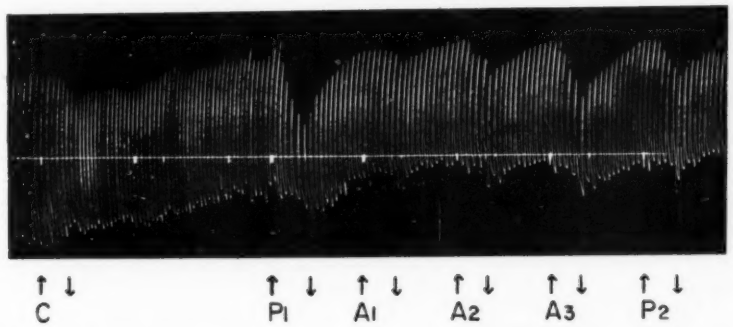
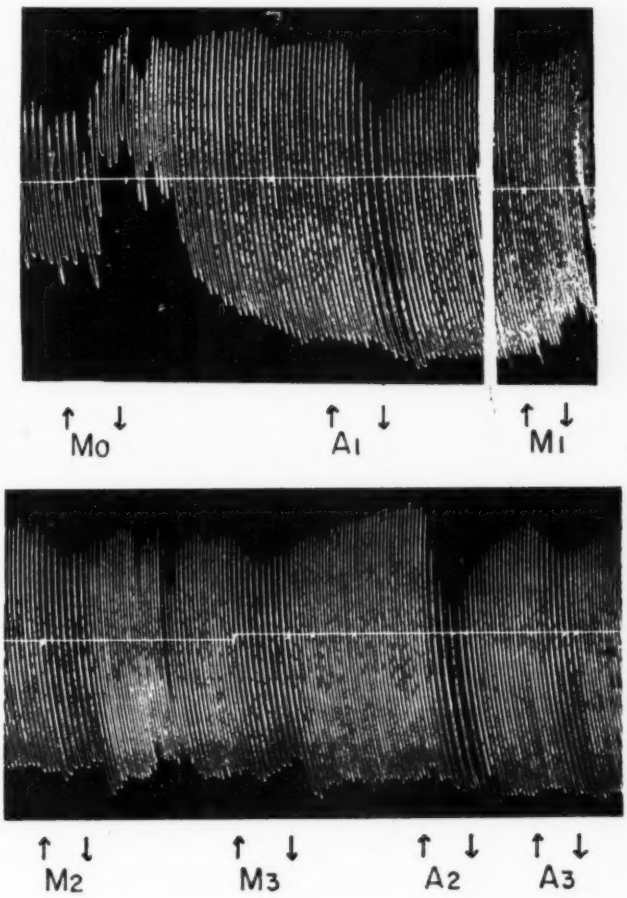


FIG. 1.—Isolated *Venus mercenaria* heart in 10 ml. bath, sensitized by previous addition of DFP ↑ indicates additions; ↓ indicates washes. At C, 1.0 ml. of whole heparinized blood (containing added paraoxone) from a rat killed by light ether anaesthesia and decapitation. The slight diminution in amplitude is accompanied by acceleration of the beat. At P_1, 1.0 ml. of whole heparinized blood (containing added paraoxone) from a rat killed by paraoxone and decapitation. The marked diminution in amplitude is accompanied by slowing of the beat. At A_1, A_2, and A_3, standard acetylcholine bromide in amounts of 0.005, 0.010, and 0.020 μg. respectively. At P_2, 0.5 ml. of the same blood sample used at P_1.

FIG. 2.—Isolated *Venus mercenaria* heart in 10 ml. bath, sensitized by previous addition of DFP. ↑ indicates additions; ↓ indicates washes. At M_0, 1.0 ml. of whole heparinized blood (containing added DFP) from a rhesus monkey anaesthetized with sodium pentobarbital. The diminution of amplitude is apparently due to failure to relax between beats, and is accompanied by acceleration of the beat. At A_1, 0.010 μg. acetylcholine bromide. At M_1, 1.0 ml. of blood from the same monkey, 30 min. after intraperitoneal injection of 1.0 mg. DFP per kg. No parasympathomimetic effect is visible. At M_2, 1.0 ml. of blood taken after a total dose of 4.0 mg. DFP per kg., and at the time of respiratory paralysis. The diminution of amplitude is accompanied by slowing, an effect typical of acetylcholine. At M_3, the same as M_2. At A_2 and A_3, 0.010 and 0.005 μg. respectively of acetylcholine bromide.



Summary

1. Lethal doses of TEPP increased the total acetylcholine content of brains of rats over that of rats killed by asphyxia, chloroform, or strychnine.

2. DFP, paraoxone, and eserine also caused an increase in total acetylcholine content of rat brain.

3. During severe anticholinesterase poisoning, free acetylcholine appeared in the blood.

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CHEMOTHERAPEUTIC INVESTIGATIONS WITH ROUS SARCOMA VIRUS

BY

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As part of a programme of investigations into the chemotherapy of virus infections, Rous sarcoma I virus has been used as one of our test viruses. This paper reports the techniques employed and the results for various substances including some known to be effective in the treatment of tumours, e.g. the nitrogen mustards, triethylene melamine, and colchicine. References to these cytotoxic agents appear in recent articles by Loveless and Revell (1949) and Rose, Hendry, and Walpole (1950), and will not be given here. Other compounds were tested because of their action on bacteriophage, e.g. R.D. 347 = 4 : 4′-bis-(2-dihydroglyoxalinyl) stilbene dihydrochloride monohydrate, and R.D. 1367 = 2 : 7-bis-(2-dihydroglyoxalinyl)-9-phenyl-phenanthridine trihydrochloride dihydrate (Dickinson and Codd, 1952), and malabar kino (Chantrill, Coulthard, Dickinson, Inkley, Morris, and Pyle, 1952). Rous sarcoma virus had been grown in fertile eggs by Murphy and Rous (1912) and Keogh (1938), and in day-old chicks by Duran-Reynals (1940); for reasons of convenience in housing animals and in the amount of drug required for treatment attention was therefore directed to the use of these hosts for testing purposes.

Experimental Methods

Strains.—The Rous sarcoma I virus was originally obtained as a freeze-dried tumour specimen from Dr. F. R. Selbie. It was passaged three times intramuscularly in fowls and once on the chorioallantoic membrane of fertile eggs. Egg membrane material was ground in broth and, after centrifugation, the supernatant fluid was filtered through a gradocol membrane of pore size 1.23 μ. This filtrate was the starting point for the work reported here.

Preparation of inocula and maintenance of stocks.—Egg membrane material was passaged 10 times on the chorioallantoic membrane of fertile eggs, 10-day embryos being used and incubation being maintained for a further 7–9 days after inoculation of the virus. Neither whole nor ground membranes maintained their titre for more than one month at $-20°$ when kept in 5 per cent glucose broth and stocks were therefore kept at $-70°$ or freeze-dried. It was later found that the livers of chicks dying from a generalized infection after the intraperitoneal inoculation of day-old chicks were much more satisfactory, for stock preparations, than either membranes or fowl tumours. Such livers were always bacteriologically sterile, were very easy to grind into uniform suspensions, and gave rise to high titre material which maintained its titre indefinitely at $-70°$ or when freeze-dried and for at least one month at $-20°$. For stocks, one liver was

ground with sand in 10 ml. lemco broth and the suspension centrifuged for 15 min. at 2,000 r.p.m. The supernatant fluid gave positive lesions in eggs at a 10^{-3} dilution and the same material, when freeze-dried, was infective at a 10^{-2} dilution. It retained its activity after gradocol filtration (1.14 μ) and was neutralized by fowl antiserum to the original Rous sarcoma.

Tests in ovo.—The technique used is described in detail by Inkley (in press). Fertile eggs from our own stock of Brown Leghorn hens were used. Fourfold dilutions of the compound under test were inoculated into the yolk sacs of groups of 10-day embryos, together with 200 u. each of penicillin and streptomycin. About 3 hr. later the virus suspension was inoculated on to the chorioallantoic membrane. Alternative drug routes, e.g. the allantoic sac and the chorioallantoic membrane, were also employed.

After a further incubation period of 7–9 days, the membranes were removed and examined for the presence of lesions. Even with the minimum of trauma and the use of a technique that gave no "non-specific" lesions with other viruses, control infected membranes did not always respond uniformly. Keogh (1938) found similar variation in individual egg response. It was therefore sometimes difficult to assess visually whether membranes with few large sarcomata were more or less heavily infected than membranes with numerous small lesions. When quantitative assessment was required, all membranes under examination were fixed in formal saline, washed in water, pressed between filter papers to remove excess moisture, and then dried in a hot-air oven at 60° for 24 hr. The dried membranes were then weighed individually and the average weight of each group was compared with the controls.

Tests in young chicks

Strains of chicks.—Brown Leghorns or Light/Heavy crosses from a reliable source were used throughout these experiments. For economic reasons light breed cockerels were generally used, but hens were occasionally substituted with similar results.

Housing and diet.—The chicks were received up to 24 hr. after hatching and put into closed metal brooders, heated by paraffin burners. Throughout the experiment the birds were kept on wire and a standard proprietary national baby chick mixture was fed *ad libitum.*

Testing of drugs.—The age of the chicks at the start of the test varied from 1–6 days as convenient. It was found preferable to use not less than 3-day-old chicks, as they had had time to adjust themselves to new conditions and normal feeding at this age. Each chick was weighed and identified by a wing band. The Rous inoculum, usually frozen or freeze-dried liver suspension, was given either subcutaneously, intraperitoneally, or intramuscularly. After a delay varying from 4 hr. to 10 days, as required, but 24 hr. in the standard test, the administration of the drugs commenced, either the subcutaneous or intraperitoneal route being used. The dose of drug was adjusted according to the body-weight of the chick (checked daily) and the range of dilution had been previously found by toxicity determinations; it included the maximum tolerated dose permitting increased growth of the chicks. The average weight gain for each complete group was calculated over a period of not less than 14 days of treatment, and expressed as a ratio of treated to control birds. Hence a non-toxic compound gave a ratio of 1.0. Chicks weighed about 40 g. at the start of the experiment, and both infected and uninfected birds increased to about 120 g. in 20 days. Since the chicks were growing rapidly, even the slightest toxic effect was apparent. Deaths in the early stages of the test were very few and all are shown in the tables of results; they included deaths due to the toxicity of the drugs as well as those of occasional weak chicks. In these experiments treatment was given once daily (except Sunday) until the experiment ended. Of the drugs employed in the tests reported here, only the antiserum was rendered sterile by filtration. The others

were considerably diluted in sterile saline and no trouble was caused by bacterial contamination. After preliminary tests the following techniques for the assessment of treatment were established for each method of infection used.

Subcutaneous.—The site chosen for the inoculum (0.25 ml.) was the skin covering the peritoneum. Drug doses were administered at the same site or directly into the peritoneal cavity. Birds were killed 9–11 days after inoculation and the parietal peritoneum was examined for the assessment of lesions ; values of 1–3 were given according to the number of lesions.

Intraperitoneal.—By inoculating into the peritoneal cavity (taking care to avoid the yolk sac) a generalized infection was produced. The virus inoculum was 0.5 ml. suspension and drugs were normally given by the same route. The spread of the disease was assessed by deaths, survival times, and the macroscopic appearance of the visceral lesions. Each bird was given a maximum value of 10 made up as follows: abdominal organs 2, parietal peritoneal membrane 2, liver 4, death 2.

Intramuscular.—The pectoral muscle was inoculated with virus suspension on either or both sides of the sternum. Drugs were given by the intraperitoneal route. The spread of the disease was assessed by the growth of the tumour (1–4), spread to viscera 4, deaths 2, giving a total maximum of 10.

RESULTS

Subcutaneous.—When the outer skin, with feather follicles, was removed at 9–11 days, the site of inoculation of the virus was exposed. The parietal peritoneum was covered with small, flat, glistening lesions similar to the discrete ectodermal proliferations produced by Rous sarcoma virus when grown on the chorioallantoic membrane of fertile eggs (Plate 1). If the birds were not killed at this stage the lesions developed into tumours, spreading along the peritoneal wall and becoming gelatinous ; eventually the tumours broke through the outer skin. On a few occasions when the birds were left on test the growth invaded the viscera and produced necrotic lesions in the liver and peritoneal cavity ; this usually happened when there was daily puncture of the peritoneum, as shown in Table II. Carr (1943) stated that chicks infected in the pectoral muscle grew tumours not only at the site of infection but also at other sites, e.g. leg muscle, where a sterile saline injection was made ; this demonstrated the tendency for tumours to grow at the site of injury. Since control birds were not usually injected daily, unlike the drug-treated ones, this fact tends to give greater significance to any prevention of tumour growth by a compound. It was not possible to remove and weigh the tumours, and assessment of results was of necessity based on the visual assessment of the lesions at 9–11 days ; values of 1–3 were obtained. On the rare occasions when there were no lesions on control membranes, lesions were observed to lie on the under surface of the membrane, presumably carried through by the puncture of the membrane in the intraperitoneal treatment.

To ascertain the nature of these lesions, a suspension of the Rous virus inoculum (liver material), was mixed with an equal volume of undiluted serum obtained from an adult fowl which had been infected a month previously with Rous virus ; injected subcutaneously in chicks this mixture produced no lesions on the parietal peritoneum (Table II). An infected peritoneal membrane, showing typical lesions, was removed aseptically from a chick ; it was ground in 10 ml. lemco broth and the suspension filtered through a gradocol membrane of pore size 1.23 μ. The filtrate,

PLATE 1.—Lesions produced by Rous sarcoma virus, 10 days after infection on the parietal peritoneal membrane of chicks. Magnification, × 2.

PLATE 2.—Appearance of the yolk sac in normal chicks. 0 = Immediately after hatching. 4 = 4 days old. 11 = 11 days old. 26 = 26 days old. One-third natural size.

which was bacteriologically sterile, was inoculated on to the chorioallantoic membrane of fertile eggs ; the lesions produced were identical macroscopically with those produced by the virus of Rous sarcoma.

Intraperitoneal.—A generalized infection was readily obtained, usually resulting in death within 21 days. Small lesions were noted, especially on the gizzard, early in the experimental period, but it was found preferable to allow the experiment to continue for three weeks, by which time most of the control infected birds were dead. The spread of the disease in the survivors was easily assessed by visual inspection of the viscera ; typical results for control birds are given in Table I. When inoculating the virus intraperitoneally, it was important to avoid the yolk sac, which occupies a large part of the abdomen in day-old chicks ; if the yolk sac were penetrated, there was no spread of infection. For this reason, apart from those reasons already mentioned, it was preferable to use chicks aged not less than about three days. If the yolk sac were pierced deaths occasionally occurred, owing to peritonitis. Another abnormality was the retention of the yolk sac ; such chicks did not gain weight normally. Plate 2 shows stages in the absorption of the yolk sac by normal uninfected chicks.

On necropsy, tumours were regularly found adhering to the parietal peritoneum, particularly at the site of inoculation. They also occurred within the peritoneal cavity, mainly on the intestinal mesentery and on the gizzard. In heavily infected controls the viscera fused into a single unit of tumour tissue, usually accompanied by excessive amounts of clear, gelatinous peritoneal fluid (at this stage death had resulted). The discrete liver lesions were 2–5 mm. in diameter, those on the surface projecting slightly ; in some birds the entire liver was necrotic and was assessed as 4, while in others only one lobe was affected. The evidence for the viral nature of the liver lesions, i.e. the effect of antiserum and filtration, has been given earlier in the paper. The lesions do not appear to be the same as those described by Duran-Reynals (1942), but we have also observed the haemorrhagic lesions in the liver reported by this author.

Intramuscular.—The tumours resulting from intramuscular injection were not easy to remove or measure for assessment. Spread into the lower peritoneal cavity occurred only occasionally, but the liver was often involved. Because of these disadvantages this route of infection was discarded after the early experiments.

Drug action on Rous sarcoma infections in eggs and chicks

Antiserum.—Antiserum to Rous sarcoma virus suppressed the development of lesions on the chorioallantoic membrane when given one hour before or immediately after the virus. It had no effect when given by the yolk sac, allantoic sac, or when given one hour after the virus on the membrane ; by this time the virus had presumably been absorbed by the cells. Similarly, antiserum suppressed the lesions on the parietal peritoneal membrane when given together with the virus. The intraperitoneal results, although not conclusive, followed the same trend. Antiserum had no effect when given daily, commencing 24 hr. after infection.

Triethylene melamine.—This compound was inactive *in ovo,* at the maximum dose tolerated by the embryo (0.01 mg.), when given via the yolk sac or via the chorioallantoic membrane. The chick results, which are summarized in Tables I,

TABLE I

DRUG ACTION ON ROUS SARCOMA INFECTION IN DAY-OLD CHICKS

Intraperitoneal route of infection, followed by intraperitoneal treatment

Dose of drugs	Delay between treatment and infection	Average gain in weight. Treated/control†	No. of chicks	Non-specific deaths or toxic deaths	Individual assessments of lesions. . Italics denote deaths	Average assessment of lesions	Value of "p"
Triethylene melamine							
0.3 μg./g. for 17 days	4 hr.	0.87_{14}	6	1	*4*, 2, 1, 3, 1, 1	2.0	<0.001
Controls	—		7	2	4, *8*, *8*, *9*, *8*, *8*, 5	7.14	
0.3 μg./g. for 16 days	24 hr.	1.0_{17}	7	1	1, 1, 2, 0, 1, 1	1.0	<0.001
Controls	—	—	10	0	*8*, 1, 0, 4, 7, 2, 1, *5*, *9*, 3	4.0	
0.3 μg./g. for 20 days	24 hr.	0.83_{14}	11	3	1, 0, 1, 2, 1, 1, 2, 0	1.0	<0.001
Controls	—		9	1	*8*, *9*, 3, *8*, 5, *9*, 2, 2	5.75	
0.3 μg./g. for 14 days	5 days	—	8	1	2, *8*, 2, *7*, 2, 0, 7	4.0	<0.001
0.3 μg./g. for 9 days	10 days	—	10	1	3, 7, *6*, 2, 6, 1, 4, 3, 8	4.4	<0.001
Controls	—	—	7	0	2, *8*, *9*, *9*, *9*, *10*, *10*	8.14	
Colchicine							
0.6 μg./g. for 3 days	24 hr.	—	12	5*	Test discontinued		
0.3 μg./g. for 20 days	24 hr.	—	8	3*	2, 3, 0, 1, *9*	3.0	<0.001
0.15 μg./g. for 20 days	24 hr.	1.0_{6}	6	0	0, 4, 2, 1, 3, *3*	2.2	<0.001
Controls	—	—	5	0	*8*, *8*, *9*, *7*, *9*	8.2	
Nitrogen mustard (bis-compound)							
0.15 μg./g. for 14 days	24 hr.	0.85_{14}	11	1*	2, *8*, *8*, *9*, 0, *8*, *9*, 0, *7*, *10*	6.1	0.05–0.02
Controls (saline for 14 days)	24 hr. 24 hr.		11	1	*8*, *9*, *9*, *7*, 6, 0, *9*, *10*, *8*, 5	7.1	
Nitrogen mustard (tris-compound)							
0.15 μg./g. for 14 days	24 hr.	0.716_{14}	11	2*	2, 2, *7*, *7*, *5*, *9*, 2, 6, 1	4.5	<0.001
Controls as for the bis-compound						7.1	
Malabar kino concentrate							
30 μg./g. for 16 days	24 hr.	0.94_{17}	10	0	*4*, 6, 4, 2, *5*, 3, 4, 2, 5, 3, *6*	3.95	
Controls as for triethylene melamine (2nd test)						4.0	
Rous antiserum							
1 dose of 0.5 ml. of 1/10 serum ..	0	—	6	0	*8*, 4, 0, *10*, *7*, 0	4.83	0.05–0.02
0.5 ml./chick/day for 9 days	24 hr.	—	6	0	*8*, 6, *8*, *4*, *10*, *7*	7.2	
0.5 ml./chick/day for 19 days	24 hr.	—	6	0	*10*, *8*, *9*, *9*, *7*, 3	7.7	
Controls	—	—	7	1	*10*, *7*, 0, *9*, *10*, *7*	7.2	

* Death due to toxicity of compound.

† Suffix to ratio refers to the period in days over which the average weight gain was calculated.

TABLE II

DRUG ACTION ON ROUS SARCOMA IN CHICKS (SUBCUTANEOUS ROUTE OF INFECTION)

Treatment commenced 24 hr. after infection, except in the case of the antiserum, which was given with the virus

Drug	Route of drug	No. of chicks	Non-specific deaths	Birds examined 9–11 days after infection for lesions on parietal peritoneum		Birds examined 20 days after infection		
				Individual assessments of lesions	Average assessment	Individual assessments of generalized infection	Average	Value of "p"
Triethylene melamine								
0.3 μg./g. ..	S.C.	12	0	1, 1, 0, 0, 0, 0, 0, 0, 0, 1, 0, 0	0.25			
	I.P.	13	0	2, 3, 0, 0, 1, H	1.2	2, 4, 2, 5, *9*, 4, 4	4.3	0.3
Colchicine								
0.15 μg./g.	S.C.	12	1*	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0	0.0			
	I.P.	13	2	0, 0, 0, 0, 1	0.2	0, 5, 0, 1, 1, 0	1.2	<0.001
Saline controls								
	S.C.	12	1	3, 3, 2, 1, 3, 1, 3, 3, 1, 3, 1	2.18			
	I.P.	13	2	2, 2, 1, 0, 2	1.4	2, 2, *9*, 4, *10*, 3	5.0	
Nitrogen mustard (bis)								
0.15 μg./g.	S.C.	11	0	1, 1, 0, 1, 1, 1, 0, 1, 2, 2, 0	0.9			
	I.P.	11	0	3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3	3.0			
Nitrogen mustard (tris)								
0.15 μg./g.	S.C.	11	1	2, 2, 0, 1, 1, 1, 0, 2, 2, 0, 0	1.1			
	I.P.	11	0	3, 3, 3, 1, 3, 1, 3, 3, 3, 3, 3	2.6			
Rous antiserum mixed with virus								
	S.C.	5	0	0, 0, 0, 0, 0	0.0			
Saline controls								
	S.C.	11	0	3, 3, 3, 3, 3, 3, 3, 3, 3, 3, 3	3.0			
Malabar kino concentrate								
30 μg./g. ..	S.C.	12	0	1, 1, 1, 1, 1, 1, 1, 3, 1, 1, 1, 1	1.2			
	I.P.	12	2	3, 3, H*, 3, 3,	3.0	*10*, *10*, 5, *10*, 6	8.2	
Controls as for colchicine and triethylene melamine								
	S.C.				2.18	—		
	I.P.				1.4	—	5.0	
R.D. 347								
3 μg./g. ..	S.C.	5	0	2, 1, 2, 3, 2	2.0			
	I.P.	5	0	2, 2, 2, 3, 3	2.4			
R.D. 1367								
3 μg./g. ..	S.C.	5	0	3, 3, 2, 3, 2	2.6			
	I.P.	5	1	1, 2, 2, 3, 2	2.0			
Controls ..	—	10	0	0, 3, 1, 0, 3, 2, 2, 3, 3, 2	1.9			

* Toxic deaths. H = haemorrhagic membrane, not assessed.

II, and III, indicate a considerable suppressive effect, at a non-toxic dose of 0.3 μg./g., on the progress of the disease, even when treatment was delayed for several days after infection. The effect was marked when both drug and virus were given by the same route, i.e. both subcutaneously. When both were given intraperitoneally there was a significant effect, but the treated animals were still heavily infected. In the range of dilutions tested, 3.0 μg./g. was toxic, causing 7/12 deaths within 10 days, 0.6 μg./g. was as active as 0.3 μg./g. and also non-toxic, but 0.15 μg./g. was much less active. When the infection was subcutaneous or intramuscular and the treatment intraperitoneal the action was much less significant.

TABLE III

INTRAMUSCULAR INFECTION FOLLOWED BY INTRAPERITONEAL TREATMENT

Dose of drug	Delay between infection and treatment	Average gain in weight	No. of chicks	Non-specific deaths	Individual assessment of lesions	Average assessment of lesions	Value of "p"
Triethylene melamine							
0.3 μg./g. for 20 days ..	24 hr.	—	11	1	1, *10*, *10*, *7*, 3, *6*, *10*, *2*, *7*, *10*	6.6	<0.001
Controls ..	—	—	11	1	*10*, *10*, *9*, *7*, *8*, *10*, *10*, *10*, *10*, 5	8.9	

Colchicine.—Colchicine was very toxic to the chick embryo and was inactive at the maximum, tolerated dose (0.0025 mg.), when given by the yolk sac.

Colchicine was the most active compound we tested in chicks. At a dose well tolerated by the chicks and allowing a good gain in weight there was complete suppression of the lesions on the parietal membrane whether the drug was given by the same route as the virus or by a different one (Table II). The results for intraperitoneal infection and treatment were very satisfactory (Table I).

Nitrogen mustards (*bis- and tris-chloroethylamines*).—The bis-compound was inactive *in ovo*, when given by the yolk sac or via the chorioallantoic membrane (0.01 mg.). The tris-compound was not tested.

Both the bis- and tris-compounds, given subcutaneously, had a slight action on the lesions on the parietal peritoneum ; they were inactive when given intraperitoneally. When both infection and treatment was by the intraperitoneal route the tris-compound was significantly active but the bis-compound was much less so. It was noted that, although the chicks gained weight, some liver damage and an occasional death occurred.

Malabar kino was inactive *in ovo* (2 mg.) and inactive in chicks infected intraperitoneally. By the subcutaneous route, the dose employed caused considerable tissue damage, the tannins in the extract being precipitated.

R.D. 347 was inactive *in ovo* (1.57 mg.) and inactive in the subcutaneous chick test at 3 μg./g.; 6 μg./g. gave rise to local tissue damage and results are not reported.

R.D. 1367 was inactive *in ovo* (1.33 mg.) and inactive in the subcutaneous chick test at 3 μg./g.; 6 μg./g. gave similar toxic symptoms to R.D. 347.

Discussion

In this work we have used Rous sarcoma as a virus typifying the tumour-producing group of viruses. It should be stressed that it is not known whether the continued presence of virus, either free or bound, is necessary for the neoplastic nature of the cells or whether the initial infection of the first cell acts as a "trigger mechanism." Consequently one must keep two chemotherapeutic aspects in mind: (*a*) the antiviral action, and (*b*) the anti-tumour action. Rous sarcoma is not commonly used as a test tumour in cancer chemotherapy. The problem was approached by us from the virus aspect, but it was obviously of interest to test known anti-tumour compounds. Fertile eggs are widely used in the chemotherapy of virus infections, but it was felt that the egg test might not respond to cytotoxic agents. Furthermore, the response of the chorioallantoic membrane to Rous sarcoma virus is not as uniform as to other viruses, so that only highly active compounds would be detected by this test. The chick test was therefore developed, and during investigations into the most suitable route of infection it was noticed that the lesions on the parietal peritoneum appeared at about the same time, i.e. 9–11 days, as on the chorioallantoic membrane. The suspicion that these were viral lesions was confirmed by the action of antiserum and the production of typical Rous sarcoma lesions when a gradocol filtrate of the ground parietal peritoneal membrane was inoculated on to the chorioallantoic membrane of fertile eggs.

The intraperitoneal route of infection gave rise to a generalized infection, large amounts of virus being recovered from the liver. The intramuscular infection gave a typical fast-growing tumour which extended to the viscera if allowed to progress beyond a certain time. In adult fowls this dissemination does not often occur and it is possible that antibodies limit the spread. Antibodies were not detected in chicks bled at 20 days, when tested on the chorioallantoic membrane by a neutralization test.

Apart from antiserum, which must be given before the virus has been adsorbed on the cells, no compounds were found active in eggs at a dose tolerated by the embryo for the 7–9 days' duration of the test. However, triethylene melamine, colchicine, and to a slight extent the nitrogen mustards had some action on the lesions of the parietal peritoneum of chicks, where a larger amount of drug could be tolerated than in the chick embryo. R.D. 347 and R.D. 1367 were quite inactive and malabar kino caused such damage to the parietal peritoneal membrane that the reduced assessment was not considered significant. It was inactive in the intraperitoneal test. Only colchicine suppressed the lesions completely and it was also the only compound to suppress them when given by the intraperitoneal route. It is possible that triethylene melamine and the nitrogen mustards did not reach the required concentration in the parietal peritoneal membrane when given by the intraperitoneal route. The results for the intramuscular infection with intraperitoneal treatment confirmed this view.

Colchicine, at 0.15 μg./g., was effective when the intraperitoneal route of infection was used, but it should be stressed that 0.3 μg./g. was toxic. Triethylene melamine was significantly active in repeated tests, even when given several days after infection; for this drug the therapeutic index was between 4 and 10. The nitrogen mustards were much less active and also toxic. As expected, antiserum was inactive

when given 24 hr. after infection. Even when given immediately after infection there was little effect ; this, however, was expected, since the antiserum was considerably diluted and dispersed in the peritoneal cavity as opposed to the subcutaneous route.

Malabar kino was included in this test merely as an example of a toxic drug. In general one would not consider, as a possible anti-virus drug, any compound markedly toxic to the host cells. Unfortunately, only cytotoxic agents have shown activity in our tests. There are as yet no known anti-virus drugs, except the newer antibiotics, which are only active against the larger viruses ; this has limited the work considerably. R.D. 347 and R.D. 1367, although active against bacterial viruses, are very toxic to animals ; they were included because the test on the parietal peritoneum seemed to offer the best chance of any activity being detected. The activity of these two drugs is not inhibited by protein, unlike that of malabar kino, where proteins precipitate the active principle, presumably a tannin.

The fact that antiserum can prevent the development of infection, if given before or immediately after infection, indicates that the chick test would be expected to pick out a compound acting against the virus as well as those acting against the tumour cell metabolism. With the cytotoxic agents the chick test revealed activity which was not detected by the egg test. Moreover, the subcutaneous route of infection enables results to be assessed almost as quickly as in the egg. The technique is simple and is proving satisfactory as a screening test.

No detailed pathological or histological studies were made in this work, in view of the approach to the problem as a chemotherapeutic study of a *virus* infection, using a typical tumour-producing virus.

Conclusions

1. Techniques for the chemotherapeutic studies of Rous sarcoma virus are described, using fertile eggs and young chicks. In the latter, viral lesions can be assessed on the parietal peritoneal membrane at 10 days.

2. Only antiserum, given before or immediately after infection, was active in the egg test.

3. Cytotoxic agents, particularly triethylene melamine and colchicine, were active in the chick tests ; the nitrogen mustards were less active.

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THE ROLES OF CORTISONE, DESOXYCORTICOSTERONE, AND ADRENALINE IN PROTECTING ADRENALECTOMIZED ANIMALS AGAINST HAEMORRHAGIC, TRAUMATIC, AND HISTAMINIC SHOCK

BY

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The secretions of the adrenal cortex play an undisputed role in the defence of the organism against all sorts of noxious agents (Swingle and Remington, 1944). Adrenalectomy renders an animal much more susceptible to a variety of traumatic procedures, as well as to the injection of endogenous or exogenous toxins, all factors which in the end have a damaging effect upon the peripheral vascular system. The adrenalectomized animal, when submitted to these noxious agents, eventually dies in circulatory failure, which can be attributed to a collapse of the peripheral circulation (Wyman and Tum Suden, 1939 ; Selye, 1949 ; Halpern and Wood, 1950a, b).

Treatment with whole cortical extracts or with desoxycorticosterone (Swingle and Remington, 1944) restores to the adrenalectomized animals at least part of their natural resistance to various forms of stress. Many hypotheses have been advanced to explain the mode of action of these hormones in protecting animals against stressing agents, but as yet no definite evidence substantiating these hypotheses has been reported. Moreover, it is not clear whether any one hormone is primarily responsible for this protection, or whether desoxycorticosterone as well as the glucocorticoids are equally able to restore to the adrenalectomized animal its natural resistance. These considerations led us to investigate whether cortisone exhibits a similar protective action against various forms of vascular shock such as haemorrhagic, traumatic, or histaminic shock, and whether the protective actions of cortisone and of desoxycorticosterone are comparable. A study of the mechanism by which the protection of these hormones is effected has been attempted.

HAEMORRHAGIC SHOCK

METHODS

The experiments were performed on white male rats weighing 125±25 g. The adrenal glands were removed in one stage by the dorsal route under light ether anaesthesia. Immediately after the operation, the animals received 1.5 mg. desoxycorti-

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costerone, and 2 ml. isotonic saline subcutaneously. They were allowed access to a normal diet with plenty of fluids. Mortality due to the operation was small (less than 10 per cent).

The haemorrhages were carried out three days after the adrenalectomy. Repeated bleedings of 0.5 ml. blood (with a maximum number of nine each) were carried out every five minutes by puncture of the ophthalmic venous plexus with a calibrated glass pipette through the inner angle of the eye (Halpern and Pacaud, 1951). The number of bleedings performed was proportional to the weight of the animals and was calculated so that the total amount of blood taken was equal to about 50 per cent of the theoretical blood volume. A number of control animals died before this amount could be withdrawn. Into all the animals who survived this procedure, the total amount of blood which had been withdrawn and heparinized was slowly reinjected intravenously. The animals were observed in the hours and days that followed and the number of deaths recorded. The animals treated with cortisone acetate or desoxycorticosterone received 5 mg. of drug the day before they were bled and again four hours before bleeding.

The rectal temperature was taken and recorded regularly throughout the experiment. The haemoglobin concentration of the blood was measured at intervals during the haemorrhage in order to ascertain the shift of fluids from the extracellular to the vascular compartment.

In order to investigate the effect of the bleedings on the arterial blood pressure, this was recorded in the carotid artery in a number of animals. The animals were anaesthetized lightly with ether or with urethane (100 mg. per 100 g.) and a small cannula introduced into the carotid artery. The haemorrhages were then carried out as described above.

Results

Control animals.—The results of these experiments are presented in Fig. 1. Out of ten control animals, only one survived for a few hours the full bleeding, which corresponded to 50 per cent of its theoretical blood volume, in spite of being transfused with the total volume of blood removed. The nine other animals died in the course of the bleedings, starting after the fourth bleeding.

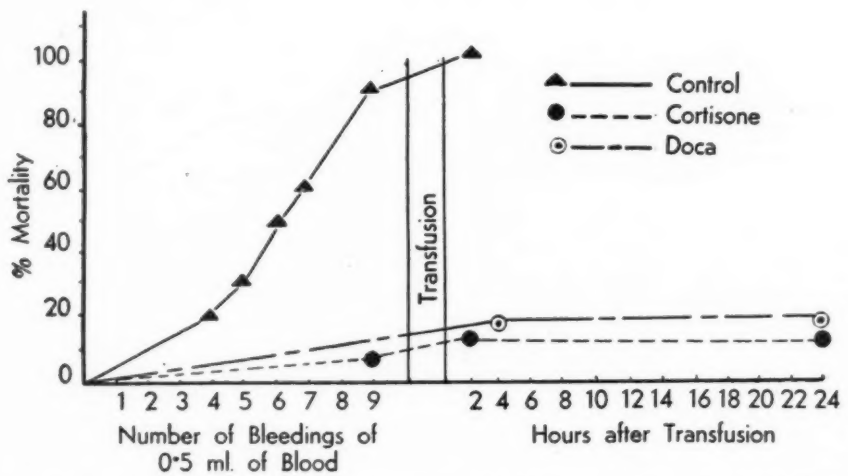

Fig. 1.—Effect of cortisone or desoxycorticosterone (Doca) on the mortality from graded haemorrhage involving 50 per cent of their blood volume in adrenalectomized rats.

All the control animals exhibited a progressive drop in rectal temperature during the bleedings as shown in Fig. 2 ; when an animal survived all the bleedings, its temperature fell to 30° C.; obviously, when a rat died in the early stages of the experiment, the fall of temperature was less striking.

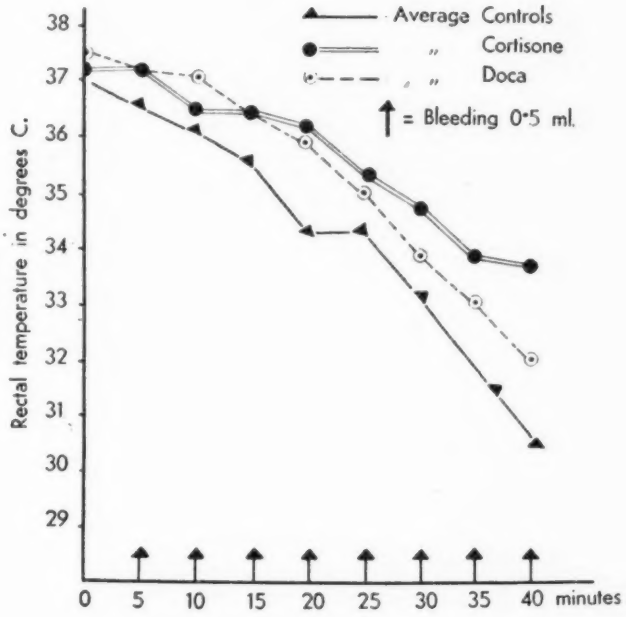

FIG. 2.—Effect of cortisone or desoxycorticosterone (Doca) on the rectal temperature of adrenalectomized rats when they sustain a graded haemorrhage involving 50 per cent of their blood volume.

Measurements of the haemoglobin concentration in the blood during the experiment are presented in Fig. 3. They indicate that in the early stages there is a slight haemoconcentration, corresponding presumably to the call on blood reserves, but in later stages there is a slight haemodilution, caused probably by absorption of fluid from the extracellular compartment.

Two typical records of the blood pressure after each bleeding are presented in Fig. 4. Generally, the successive bleedings produce a fall of blood pressure, which is relatively sharp at the time of the bleeding. The fall of blood pressure between two successive bleedings is usually 1 to 2 cm. Hg. Usually, after the seventh or eighth bleeding, the animals die suddenly owing to vascular collapse. Two animals maintained a relatively high blood pressure until the fifth bleeding, but it then fell progressively and severely. Two animals, who survived nine bleedings and were transfused with their own blood, showed a return of blood pressure to a nearly normal value, but could not maintain it and, after a fresh fall of blood pressure, died within an hour.

Animals treated with cortisone.—Out of fourteen treated animals, none died before the total amount of blood had been withdrawn ; one died after the ninth bleeding, and another one died two hours after being transfused ; twelve animals were alive after twenty-four hours, and may be considered as having definitely recovered (Fig. 1).

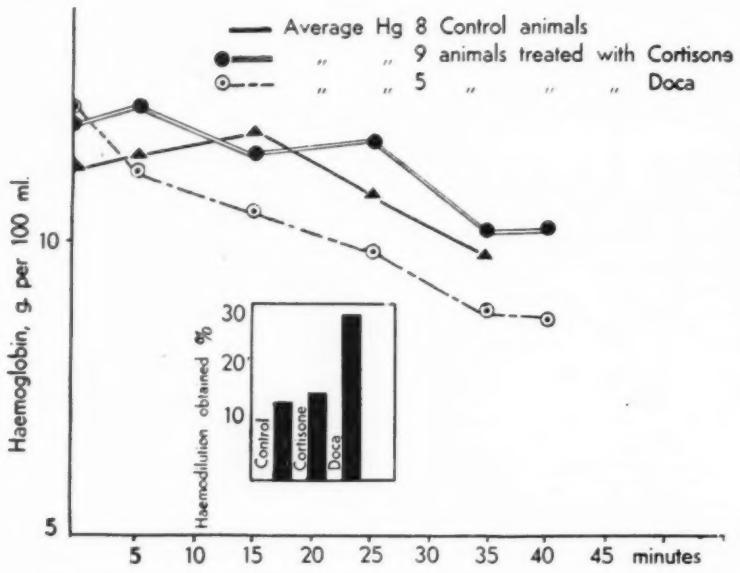

FIG. 3.—Effect of cortisone or desoxycorticosterone on haemoglobin concentration of the blood and haemodilution in adrenalectomized rats while they sustain a graded haemorrhage involving 50 per cent of their blood volume.

It must be stressed (see Fig. 2) that animals treated with cortisone tend to maintain their body temperature much more efficiently than the controls. A study of the haemoconcentration curves (Fig. 3) reveals no significant difference between cortisone-treated animals and the controls. However, analysis of the blood-pressure records (Fig. 4) shows a strikingly different behaviour between cortisone-treated and control animals. The blood pressure of the treated rats is generally higher by an average of 1.5 cm. Hg than that of the controls. During the haemorrhages the fall of blood pressure in these animals seldom becomes significant before the fifth bleeding. After this, when it starts to drop, the slope of the blood pressure is much less steep than in the control animals. Arterial pressure never falls to very low levels, and after transfusion the blood pressure rises and is maintained at a normal level.

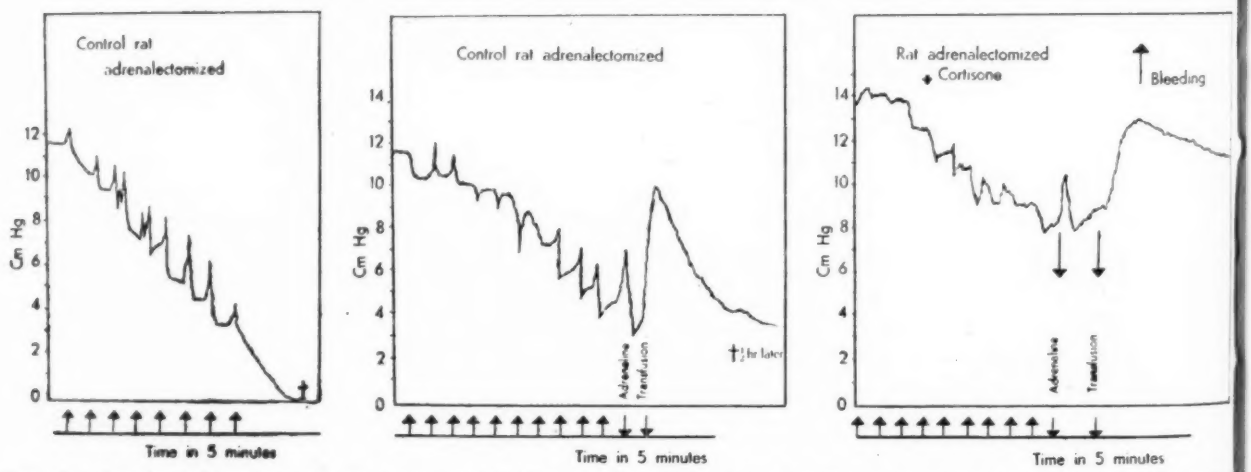

FIG. 4.—On the left and in the middle: rapid drop of the arterial blood pressure in control rats during the course of the bleedings. On the right: record of blood pressure in a cortisone-treated rat who sustained the same haemorrhagic procedure and was transfused after the ninth bleeding.

Animals treated with desoxycorticosterone.—Out of six treated animals, one died a few hours after being transfused, and the remainder survived twenty-four hours and may be considered as having definitely recovered. The rectal temperatures of these animals were, on the average, higher than those recorded for the control group, but lower than those of the cortisone-treated animals (Fig. 2).

Study of the haemoconcentration (Fig. 3) reveals a significant difference between the rats treated with desoxycorticosterone and those treated with cortisone and the controls. Even in the early stages there is no haemoconcentration, but rather a progressive haemodilution which in the end reaches an average of 28 per cent of the original haemoglobin concentration; in the control and the cortisone-treated animals, on the other hand, it reached an average value of only 14 per cent. There is, in these animals, a greater transfer of fluids from the extracellular spaces into the vascular bed, which much improves the chances of recovery. The blood pressures, in this group, during the haemorrhages were not very different from those of the control rats, except that, after transfusion of the withdrawn blood, the animals treated with desoxycorticosterone maintained their normal blood pressure much better than the control animals, but not quite as well as those treated with cortisone.

Limb Ischaemia Shock

Methods

These experiments were performed on 51 rats three days after adrenalectomy; the rats were adrenalectomized and treated as described above. The animals treated with cortisone acetate or with desoxycorticosterone received subcutaneously 5 mg. of the drug the day before the application of the tourniquet. Another 5 mg. was administered two hours before application of the garrotte, and 1.5 mg. six hours after the removal of the tourniquet.

Rubber tourniquets were applied at the origin of the thigh of the left leg in one group of animals, and to the same site on both legs in another group; they were maintained for two hours, the animals being kept in separate cages. The tourniquets were then removed and the limbs massaged gently. The animals were kept in a well-heated room. Haemoconcentration was measured from the change in haemoglobin concentration.

Results

The results of these experiments are presented in Table I. It can be seen that, when the tourniquet was applied to one limb, 7 out of 10 controls died in the 12

TABLE I

EFFECT OF CORTISONE AND OF DESOXYCORTICOSTERONE ON MORTALITY FROM LIMB ISCHAEMIA IN ADRENALECTOMIZED RATS

Limb ischaemia One limb—2 hours Mortality at 24 hours			Limb ischaemia Two limbs—2 hours Mortality at 24 hours		
Control	After cortisone	After desoxycorti-costerone	Control	After cortisone	After desoxycorti-costerone
7/10	0/11	0/6	11/12	0/10	0/7

hours that followed the removal of the tourniquet. All the animals treated with cortisone or with desoxycorticosterone survived. When the tourniquets were applied to two limbs, 11 out of 12 control animals died, and again all the animals treated with cortisone or desoxycorticosterone survived.

The changes in haemoconcentration in the control animals and in those treated with cortisone when the tourniquet was applied to one limb were studied. No significant difference between the control and the cortisone-treated animals was apparent. It should be noted that the maximum haemoconcentration observed was relatively small, and far from sufficient to explain the death of the control animals on the basis of loss of fluid in the limb.

Histaminic Shock

It is well known that mice are relatively insensitive to histamine. According to Halpern and Wood (1950a, b), the lethal dose of histamine dihydrochloride, injected intraperitoneally, is 50 mg. per 20 g. of body weight for normal mice. Adrenalectomy renders mice considerably more sensitive to histamine, so that it requires only 0.5 mg. per 20 g. of histamine to kill an adrenalectomized animal (Halpern and Wood, 1950b). It has been shown by the same authors that adrenaline increases the resistance of the adrenalectomized mice to histamine, but only raises the lethal dose of histamine from 0.5 mg. to 5 mg. per 20 g. Adrenaline therefore does not seem to be the only factor responsible for the high resistance of normal mice to histamine.

Methods

One hundred and twenty-four adult mice (female) weighing about 20 ± 5 g. were used for these experiments. Bilateral adrenalectomy was performed under light ether anaesthesia and the animals were allowed access to a normal diet with plenty of fluid. Immediately after the operation they received 0.5 mg. desoxycorticosterone and 0.5 ml. isotonic saline subcutaneously. The animals treated with cortisone acetate or desoxycorticosterone were injected with 0.5 mg. of drug twice a day for three doses, the last dose being given 4 to 5 hours before the injection of histamine. Histamine dihydrochloride was administered intraperitoneally in 0.5 ml. isotonic saline per 20 g. of body weight 48 hours after the operation. Two groups of animals treated with cortisone or desoxycorticosterone were also treated with adrenaline. To these animals and to a number of controls, 20 μg. adrenaline per 20 g. body weight was injected subcutaneously 30 min. before the injection of histamine. In some animals, haemoconcentration was measured by the change in the haemoglobin concentration of the blood. As in the rat, blood samples were obtained by puncture of the ophthalmic venous plexus through the inner angle of the eye with a capillary glass pipette (Halpern and Pacaud, 1951).

Results

The results of these experiments are presented in Table II and in Fig. 5. They show that cortisone can increase the tolerance of adrenalectomized mice to histamine from 0.5 mg. to 2.5 mg. per 20 g., whereas desoxycorticosterone is not capable of protecting the animals against one lethal dose of histamine. This protective power of cortisone, however, does not restore to the animals their ability to withstand doses of histamine tolerated by normal mice. In contrast to this weak protective power of cortisone alone, treatment with both adrenaline and cortisone

TABLE II

EFFECT OF CORTISONE, DESOXYCORTICOSTERONE, AND ADRENALINE ON HISTAMINE TOXICITY IN ADRENALECTOMIZED MICE

Histamine mg./20 g.	Control	After adrenaline	After cortisone	After desoxycorticosterone	After desoxycorticosterone and adrenaline	After cortisone and adrenaline
0.25	3/9			1/3		
0.5	10/10		0/10	4/6		
2.5			5/14	4/4		
5.0		1/3	10/15			
10.0		4/5			2/8	0/6
17.5					2/5	
25.0					4/5	2/15
50.0						7/7

confers on adrenalectomized mice the ability to withstand the same doses of histamine as normal mice. It should also be noted that, although desoxycorticosterone has practically no protective power by itself, since it cannot protect against one lethal dose of histamine, it is able to increase considerably the antihistaminic power of adrenaline in the adrenalectomized mouse, although to a much lesser degree than cortisone.

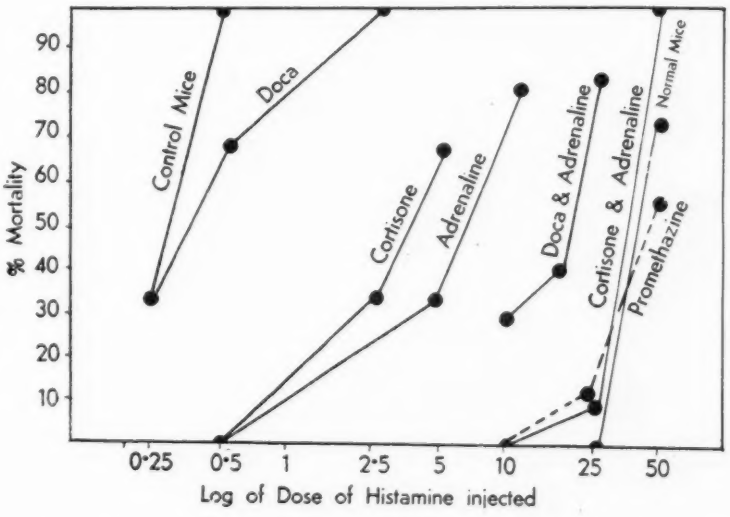

FIG. 5.—Effect of cortisone, desoxycorticosterone (Doca), and adrenaline on the toxicity of histamine in adrenalectomized mice. The data concerning promethazine (dotted line) have been extracted for comparative purposes from the study by Halpern and Wood (1950b).

The degree of tolerance to histamine afforded by cortisone and adrenaline is reflected in the degree of haemoconcentration (Table III). When the animals are protected with cortisone, or cortisone and adrenaline, the degree of haemoconcentration which usually results from the injection of histamine diminishes. Thus the degree of haemoconcentration observed in adrenalectomized control animals, who received 0.5 mg. per 20 g. histamine, was 65 per cent. In animals treated with cortisone, the same dose of histamine produced an increase of haemoconcentration of only 12 per cent; when adrenalectomized mice were treated with cortisone, a

TABLE III

EFFECTS OF CORTISONE AND ADRENALINE ON HAEMOCONCENTRATION PRODUCED BY HISTAMINE IN ADRENALECTOMIZED MICE

Second blood sample taken 20–30 min. after histamine injection. Haemoconcentration measured from change in haemoglobin concentration.

Treatment	Average haemoconcentration per cent after histamine 2 HCl (mg.)					
	0.25	0.5	2.5	5.0	10	25 mg.
Control mice	31	65				
After 20 μg. adrenaline HCl ..				25	47	
After cortisone		12	31	50		
After cortisone + 20 μg. adrenaline HCl ..					2	8

dose of histamine as high as 2.5 mg. per 20 g. brought about an increase of haemoconcentration of 31 per cent. Moreover, when the animals were treated with both cortisone and adrenaline, they tolerated as much as 25 mg. histamine per 20 g., and the average haemoconcentration was only 8 per cent.

Discussion

The results reported above indicate that cortisone provides the adrenalectomized animal with a definite protection against haemorrhagic shock. Whereas control animals die early with symptoms of vascular collapse, treatment with cortisone makes it possible for adrenalectomized animals to survive repeated bleeding and to maintain relatively high blood pressures and body temperatures during the procedure. This protective effect is probably due to an improvement of the vascular tone of the small capillaries and arterioles, the animals being able to maintain, in spite of drastic reductions of blood volume, arterial blood pressures sufficient for the safeguarding of essential life processes. It is well known that the arterioles of adrenalectomized animals are not able to compensate for loss of blood and show a decreased sympathetic tone (Swingle and Remington, 1944 ; Wyman and Tum Suden, 1939) ; considerable evidence, both direct and indirect, indicates that the capillaries of the adrenalectomized animal in insufficiency are atonic, dilated, and abnormally permeable (Wyman and Tum Suden, 1939 ; Zweifach and Chambers, 1942 ; Selye, 1949).

Treatment with cortisone restores normal function to these vessels in adrenalectomized animals. It should be noted that the animals treated with cortisone generally exhibit a higher blood pressure than the control animals, and this can also be interpreted as being due to an increase of vascular tone.

Treatment with desoxycorticosterone also protects the animal against death from haemorrhagic shock, but the effects on the blood pressure, the temperature, and the changes in haemoconcentration are somewhat different than those seen in animals treated with cortisone. Desoxycorticosterone does not maintain either blood pressure or temperature as well as cortisone does. The greater haemodilution observed in animals treated with desoxycorticosterone at the end of the bleeding could be a considerable contributing factor to survival. Desoxycorticosterone causes a water

and salt retention in the extracellular fluids, with easier transfer of fluids into the vascular compartment when the need arises.

The results obtained in the limb ischaemia experiments show that cortisone and desoxycorticosterone protect the animal equally well against death in the crush syndrome. Death in limb ischaemia is probably due to vascular collapse resulting from absorption of angiotoxic products from the ischaemic limb. The relatively small degree of haemoconcentration in these animals and the lack of difference between the haemoconcentrations of the treated and the control groups, in spite of the difference in mortality, indicate that the loss of plasma fluid is not the most important factor in the mechanism of the vascular collapse.

The absorption of toxins from the ischaemic limb can cause a massive peripheral vasodilatation, with decreased blood pressure, stasis, and vascular collapse. It is probable that the cortical hormones act here on the vascular tone, through the same mechanism as in haemorrhagic shock, without it being necessary to invoke a specific detoxifying mechanism to explain the action of the hormones. These facts should be connected with the observation that the adrenalectomized animal shows a much poorer ability to perform muscular work than the normal animal, in spite of the fact that the isolated muscles from both animals perform equally well (Ramey, Goldstein, and Levine, 1950). This decreased ability to perform muscular work has been ascribed to the asthenia of the blood vessels and the poor irrigation of the organ at work. It can be restored to normal by treatment with cortisone.

The results obtained in histaminic shock indicate that cortisone is much more active than desoxycorticosterone in its capacity to confer on the adrenalectomized animal resistance to vascular injuries.

The animals treated with cortisone can tolerate a dose of histamine about five times larger than the control adrenalectomized animals. In this respect, cortisone behaves much like adrenaline alone in these adrenalectomized animals. None of these substances alone can restore the normal tolerance to a single injection of histamine, as does promethazine (Halpern and Wood, 1950b). However, treatment with adrenaline of adrenalectomized mice, which have been receiving cortisone for 24 hours, confers on them the ability to withstand the high doses of histamine tolerated by the normal mice.

Although treatment of animals receiving desoxycorticosterone with adrenaline does not bring back the tolerance to histamine to the normal level, as it does for cortisone-treated animals, it makes it possible for the animal to withstand higher doses of histamine than does treatment with adrenaline alone.

These observations suggest that cortisone, and to a lesser extent desoxycorticosterone, maintain the tone of blood vessels by improving the ability of the vascular bed to respond to vasoconstrictor agents. It must be mentioned that Levine and his co-workers (Ramey, Goldstein, and Levine, 1951 ; Fritz and Levine, 1951) have already observed that treatment of adrenalectomized animals with cortisone increases and regulates the vascular effect of noradrenaline. This interpretation of the mode of action of cortisone on small vessels is in harmony with the numerous reports that the sensitivity to injected rennin is lost in adrenalectomized animals, and is restored only by cortical extracts (Friedman, Somkin, and Oppenheimer, 1940 ; Remington, Collings, Hays, Parkins, and Swingle, 1941). It is also consistent with the observation of Selye (1950) that adrenaline promotes only a temporary rise

in the resting blood pressure of adrenalectomized animals, and that their tendency to develop severe hypotension during stress cannot be effectively combated by this hormone. The vasoconstrictor effect of adrenaline in the mesentery of the rat similarly declines progressively after adrenalectomy.

The findings reported here indicate that cortisone plays a predominant role in the resistance of animals to traumatic and toxic injuries: cortisone seems to enhance this resistance by improving the compensatory vascular reactions which are markedly depressed during the state of shock. The results obtained in experiments involving histaminic shock suggest that cortisone, and to a lesser degree desoxycorticosterone, are potentiating the effect of adrenaline in combating the angiotoxic effects of histamine.

Summary

1. The actions of cortisone, desoxycorticosterone and of adrenaline on haemorrhagic, traumatic, and histaminic shock in recently adrenalectomized animals have been investigated.

2. In haemorrhagic shock induced in adrenalectomized rats by repeated and graded haemorrhage, cortisone, and to a lesser extent desoxycorticosterone, each produce a definite protective effect. Whereas 90 per cent of control animals die early with symptoms of irreversible vascular collapse, treatment with cortisone makes it possible for animals subjected to bleeding to survive and to maintain a relatively high blood pressure and body temperature. Desoxycorticosterone cannot maintain either the blood pressure or the body temperature so well as cortisone does.

3. In animals submitted to limb ischaemia either cortisone or desoxycorticosterone protect the animals equally well against death from crush syndrome. Animals dying from limb ischaemia die probably from vascular collapse owing to the absorption of angiotoxic products from the ischaemic tissue. The relatively small haemoconcentration found in these animals and the lack of difference in the degree of haemoconcentration between the treated and the control animals, in spite of the drastic difference in mortality, indicate that the loss of the plasma fluid is not the most important factor in the mechanism of the vascular collapse.

4. The results obtained in histaminic shock indicate that desoxycorticosterone does not modify the high sensitivity of adrenalectomized mice to histamine, whereas cortisone increases this tolerance about five times and adrenaline about ten times. None of these three hormones alone can restore the natural resistance to histamine in the adrenalectomized animal. However, in adrenalectomized mice receiving cortisone, treatment with adrenaline confers the ability to tolerate doses of histamine as great as those tolerated by the normal animal. Desoxycorticosterone associated with adrenaline has a similar effect although it does not restore the tolerance to the normal level.

5. The results indicate that cortisone and, to a lesser degree, desoxycorticosterone enhance the resistance of animals to traumatic and toxic injuries by improving the ability of the small vessels to respond to the exogenous and endogenous vasoactive agents.

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THE QUANTITATIVE DETERMINATION OF DIFFUSIBLE HISTAMINE IN BLOOD

BY

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WITH AN ADDENDUM BY FLORA J. PHILPOT

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Under physiological conditions most of the histamine in blood is apparently associated with the leucocytes (for references see Guggenheim, 1951), but it is not known in what form the histamine exists. Methods for determining the concentration of histamine in blood generally do not distinguish between histamine which is pharmacologically active and histamine which may be present in inactive combination. When histamine is extracted by chemical means combined histamine may be released (Rocha e Silva, 1944). Furthermore, histamine may be produced by decarboxylation of histidine (Äkerblom, 1941 ; Schmiterlöw, 1949), although Hughes, Salvin, and Wood (1951) have suggested that this source of error is of no practical importance in the method of Barsoum and Gaddum (1935) and its much used modification by Code (1937). Other disadvantages of published methods are the considerable amount of work involved in purifying histamine (Code, 1937 ; McIntyre, Roth, and Shaw, 1947) and the low recoveries (Roberts and Adam, 1950).

We have developed a new method because we wanted to estimate that fraction of histamine in whole blood which could be assumed to be pharmacologically active. That fraction was presumed to be represented by the histamine which was able to diffuse from blood when the blood was dialysed against physiological saline.

Tarras-Wahlberg (1935, 1936) separated histamine from blood by dialysis and Emmelin (1945a) by ultra-filtration. However, Emmelin's method required specially prepared collodion membranes.

We found that histamine in aqueous solution dialysed rapidly through commercial cellophane membranes (Fig. 1). When saline or blood containing histamine was dialysed against an equal volume of saline (see below) the concentration of histamine in the system reached equilibrium in less than one hour. Volume changes due to differences in osmotic pressure were negligible and the dialysate contained half of the original histamine.

The dialysate of blood also contained substances which interfered with the assay of histamine on the isolated ileum of the guinea-pig, and it was necessary to purify the histamine. This was done by means of paper partition chromatography. Dent (1948) and Urbach (1948, 1949) used paper chromatography to isolate histamine qualitatively.

Preliminary experiments showed that when histamine-containing dialysates of blood were acidified with dilute HCl and boiled to dryness, no histamine was lost

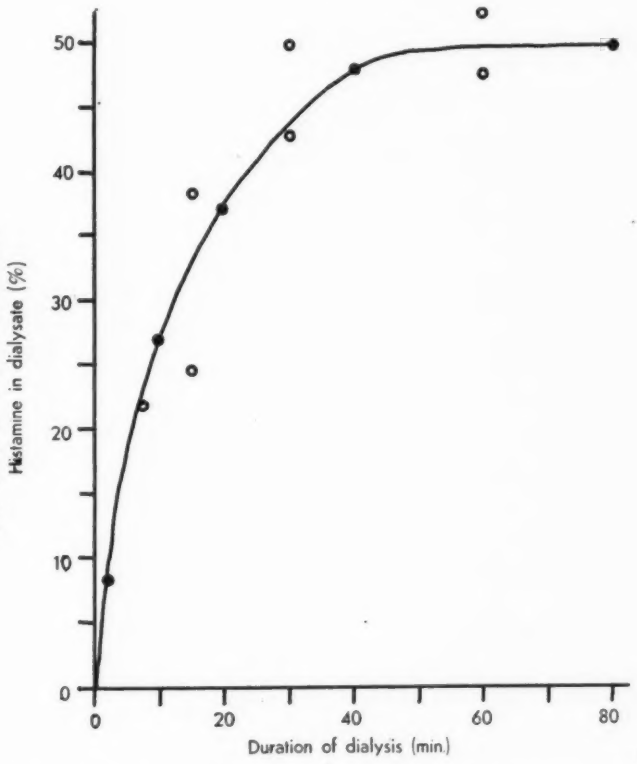

FIG. 1.—Rates of dialysis of histamine. Solid circles : A concentrated aqueous solution of histamine dihydrochloride was dialysed against an equal volume of water. The amounts of histamine which had dialysed were determined gravimetrically on samples of the dialysate. Open circles : Histamine in blood was dialysed against an equal volume of saline, as shown in the first two recovery experiments in the Table.

and none was formed from histidine (Abel and Kubata, 1919 ; Gavin, McHenry, and Wilson, 1933 ; Hughes *et al.*, 1951) or N-acetylhistamine (Roberts and Adam, 1950). Furthermore, no histamine was lost when the dried material was extracted with methanol and the extract purified chromatographically in a butanol-acetic acid system. The developed wet chromatogram showed a band (Rf 0.15–0.25) the opacity of which was distinctly different from that of the rest of the paper. It was found that this band was due to inorganic salts carried over in the methanol extraction. When the dried chromatogram was sprayed with ninhydrin to demonstrate amino-acids (Dent, 1948) and Pauly's diazo reagent to demonstrate histamine and similar compounds (Guggenheim, 1951), it was found that the band containing the inorganic salts also contained all the histamine but no other substance which reacted with these reagents or interfered with the subsequent biological assay. The histamine could be eluted from this band without loss and assayed biologically.

PROCEDURE

A sample of blood (2 ml. to 4 ml.) was taken and semicarbazide immediately added to give a concentration of 1×10^{-5} M in order in inhibit any histaminase present (Mongar and Schild, 1951).

The blood was pipetted into a cellophane bag (20/32 " Nojax " casing made by the Visking Corporation, Chicago) and dialysed against an equal volume of physiological saline in a short, wide test-tube. To ensure good mixing, the tube was stoppered with a rubber bung, clamped to a wheel, and mechanically rotated at about 15 revolutions per min. for 1 hr.

The bag was discarded and the saline solution acidified with 0.1 ml. N-HCl and evaporated to dryness in a bath of boiling water. The residue was extracted three times with about 0.4 ml. methanol, and the extracts were transferred quantitatively to a pencil line drawn on a sheet of Whatman No. 1 filter paper, prepared for chromatography as shown in Fig. 2. The methanolic solution was transferred to the pencil line with a capillary pipette and dried with a current of warm air from a hair-drier. The solution was not allowed to spread more than 0.5 cm. to each side of the line. The transfer of a sample took less than 5 min.

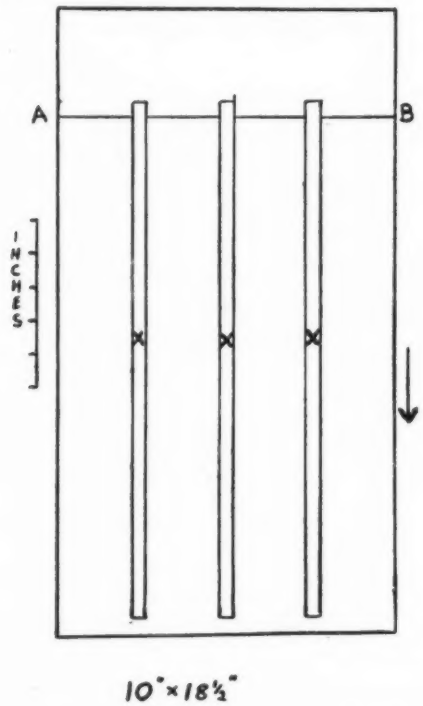

FIG. 2.—A sheet of Whatman No. 1 paper was halved to give two sheets 10 in. × 18½ in. By cutting out the pieces marked X, the paper was divided into four columns, and there was no risk of the substances in one column mixing with those in the next. The extracts of the dialysates were applied to the starting line A–B, and the chromatogram was developed in the direction of the arrow.

The chromatogram was developed overnight at room temperature with a butanol-acetic acid system as the mobile phase (i.e., the butanol phase obtained after mixing 25 vols. of *n*-butanol, 25 vols. of distilled water, and 6 vols. of glacial acetic acid). On the developed, wet chromatogram the opaque band due to inorganic salts was marked. The chromatogram was dried with a current of warm air from a hair-drier until it did not smell of butanol or acetic acid. The marked area was cut into small pieces and briefly shaken in a known volume of Locke's solution. The histamine in this solution was then assayed on the isolated ileum of the guinea-pig, in a bath of 2 ml. capacity. All histamine assays are given in terms of the free base.

RESULTS

A series of recovery experiments were made in order to test the efficacy of the method. Cats were given heparin and bled under nembutal anaesthesia ; semicarbazide was mixed with the blood. Histamine was added to the blood in known amounts and 4 ml. samples were assayed by the method described. As cat blood contained a basal level of histamine (see below) the histamine content of the blood without added histamine was also determined. The results are shown in the Table.

TABLE

RECOVERY EXPERIMENTS TO TEST THE METHOD

All amounts of histamine refer to 4 ml. blood

Dialysis time (min.)	Histamine found in blood only (μg.)	Histamine added (μg.)	Total (μg.)	Total histamine found (μg.)	Recovery (to nearest 5%)	
15	0.02	0.4	0.42	0.2	50	Same experiment
30	0.02	0.4	0.42	0.36	85	
60	0.02	0.4	0.42	0.47	110	
7.5	0.6	0.5	1.1	0.47	45	Same experiment*
15	0.6	0.5	1.1	0.80	75	
30	0.6	0.5	1.1	1.11	100	
60	0.6	0.5	1.1	1.03	95	
60	0.6	0.4	1.0	0.89	90	
60	0.6	0.8	1.4	1.34	95	
60	0.6	1.6	2.2	2.4	110	
60	0.02	0.3	0.32	0.32	100	
60	0.02	0.66	0.68	0.64	95	
60	0.02	0.87	0.89	0.84	95	
60	0.02	0.55	0.57	0.50	90	
60	0.02	1.10	1.12	1.20	105	
60	0.02	0.77	0.79	0.83	105	
60	0.02	0.44	0.46	0.46	100	
60	0.2	1.8	2.0	1.6	80	
60	0.2	4.8	5.0	5.2	105	
60	0.06	0.4	0.46	0.44	95	
60	0.06	0.4	0.46	0.40	85	
Average of recoveries for 60 min. dialysis (16 experiments)					95	

* In this experiment the blood was collected in a vessel containing heparin. The "blood only" figure was abnormally high; this was probably due to histamine in the heparin. In all the other experiments the heparin was injected into the cat 10–20 min. before the blood was taken.

It will be noted that this test of the method depended upon two determinations: (*a*) to find how much histamine was contained in the original sample of blood, and (*b*) to find how much was contained in the blood after a known amount had been added. In view of this procedure, depending upon two assays, the recovery figures were good, the lowest being 80 per cent and the highest 110 per cent.

Histamine concentration in cat's blood

In six cats anaesthetized with chloralose the concentrations of blood histamine were 5.5, 5.0, 3.1, 1.6, 1.2, and 0.9 μg./100 ml. blood. These results agree with those published by Brown, McIntosh, and White (1941), Emmelin (1945b), and Ojha and Wood (1951).

Discussion

Dialysates of blood contained substances other than histamine which acted upon the isolated guinea-pig's ileum. "Slow-contracting substances" were eliminated in the purification. Potassium estimations by means of flame photometry showed that the concentration of K ions in saline dialysates of blood or plasma was 75 μg./

ml., and of haemolysed blood, 110 μg./ml. It was found that any K present with histamine in the solutions used for assay was insufficient to affect the guinea-pig ileum or to alter its sensitivity towards histamine. A disadvantage of dialysis was that only half of the blood histamine was recovered.

Paper chromatography of pure histamine showed that the substance moved in a well-defined band. The *n*-butanol-acetic acid system was chosen because the separation of histamine (Rf 0.15) and N-acetylhistamine (Rf 0.43) was greater than when the *n*-butanol-ammonium hydroxide system of Urbach (1948) was used, and because histamine was stable in the acidic system and could be completely recovered from it. The inorganic salts in the chromatogram did not affect the mean Rf value of histamine.

As little as 1 μg. histamine in 100 ml. blood could be determined. The errors of the estimations in the recovery experiments could be accounted for by the bioassay alone. Recently, several methods have been published for the colorimetric determination of histamine (Camisasca, 1949 ; Madar, Sterne, Rosin, and Frediani, 1950 ; Graham, Lowry, and Harris, 1951). Some of them are claimed to be as sensitive as bioassay. Therefore, the present method could perhaps be improved by using colorimetry instead of bioassay in the estimation of the purified histamine.

Summary

A method is described for the quantitative determination of diffusible histamine in blood. Histamine is separated from blood by dialysis, purified by paper partition chromatography, and assayed biologically.

When histamine was added to blood and assayed by this method, all the histamine was recovered.

We wish to thank Dr. G. S. Dawes for his interest in this work, and one of us (J.R.V.) is grateful to the Medical Research Council for a personal grant.

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ADDENDUM

BY

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In the absence of salts adrenaline and noradrenaline separate clearly from histamine on a paper chromatogram in the butanol-acetic acid system (Born and Vane, 1952); Rf values: histamine 0.15, noradrenaline 0.28, adrenaline 0.36 (Shepherd and West, 1951). With increasing quantities of salts the rate of travel of adrenaline and noradrenaline is decreased. The quantity of salt which is transferred to the chromatogram in solution in methanol, as described by Born and Vane (1952), makes it impossible to free histamine from adrenaline and noradrenaline. If these are present in concentrations sufficient to interfere with the bioassay of histamine the following modification of Born and Vane's procedure is necessary. Acid ethanol is substituted for methanol in order to reduce the amount of salts transferred to the chromatogram, but since histamine itself has a low solubility in ethanol, certain precautions are necessary in order to ensure full recovery of histamine.

After evaporation of the dialysate the residue was extracted with 3×0.4 ml. methanol; 5 ml. acid ethanol (0.1 per cent (v/v) conc. HCl) were added to the extract and the whole was evaporated to about 0.5 ml. on a boiling water bath. The extract was transferred to a 2 ml. tube and the residue washed twice with 0.25 ml. acid ethanol. The extract and washings were combined, cooled to $-10°$ C., and centrifuged. The supernatant fluid was carefully withdrawn and applied to a filter paper and the chromatogram was developed as described by Born and Vane. The salt band was very small or absent, and consequently a control was necessary to indicate the position of the histamine in the chromatogram. A sample of blood was dialysed, extracted, and 5 μg. each of histamine, adrenaline, and noradrenaline were added. After chromatography the control strip was sprayed with a solution of potassium ferricyanide (James, 1948) to locate adrenaline and noradrenaline and with Pauly's reagent to locate histamine.

It was possible by this procedure to recover 100 per cent of added histamine (1 μg. – 0.05 μg./ml.) from dog's blood to which had been added adrenaline and noradrenaline, each at a concentration of 5 μg./ml.

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* I.C.I. Research Fellow.

Brit. J. Pharmacol. (1952), 7, 304.

EFFECT OF DENERVATION ON AMINE OXIDASE IN STRUCTURES INNERVATED BY THE SYMPATHETIC

BY

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(Received February 22, 1952)

The presence of amine oxidase in the blood vessels of the rabbit has been described by Thompson and Tickner (1951). Its presence in the nictitating membrane and the iris of the cat has been described by one of us (Robinson, 1952), who has also observed its presence in the blood vessels of the cat. Experiments have now been performed to see whether the amount of amine oxidase present is modified by denervation, for if amine oxidase is concerned with the destruction of the sympathetic transmitter noradrenaline, and if the degeneration of the sympathetic fibres reduced its amount, the increased sensitivity of denervated structures to noradrenaline and adrenaline might be explained.

METHODS

Observations have been made on the nictitating membrane and on the iris of the cat's eye and on vessels of the cat's foreleg. The nictitating membrane and the iris of the right side were denervated by extirpation of the superior cervical ganglion in an aseptic operation ; the vessels of the right foreleg were denervated in other cats by a similar removal of the stellate ganglion. At varying periods after the operation the amine oxidase in the organs was determined manometrically, a comparison being made of the amount present in the normal and in the denervated structures.

Control observations on all three tissues were first made to compare the two normal sides. For observations on the nictitating membrane at first two membranes were used for one experiment ; that is to say, the membranes from two right eyes were compared with the membranes from two left eyes ; later it was found possible to compare the membrane from one right eye with that from one left eye. For observations on the iris, the irises from three to seven right eyes were compared with the corresponding number from left eyes. For observations on the blood vessels, the vessels from four to six right legs were compared with vessels from the corresponding number of left legs.

Each nictitating membrane was removed immediately after death by cutting round the orbit with a scalpel, removing the eyeball from the orbit, and dissecting the membrane off the eyeball. The tissue having been weighed, a suspension was prepared by alternately cutting the tissue with scissors and pounding it in an ice-cold mortar ; 4 ml. of 0.067M-sodium monohydrogen phosphate buffer of *p*H 7.4 was added for each membrane. For further details the paper by Robinson (1952) should be consulted. Tyramine hydrochloride was used as a substrate, and the incubation was carried out in the presence of KCN in order to exclude oxidation reactions other than those due to the amine oxidase system.

Amine oxidase activity was expressed as the additional oxygen uptake in μl. per g. per hr., and this figure was determined in two ways. It was calculated from the rate of

additional oxygen uptake during the first 20 or 30 min. of the incubation, and it was also determined from the additional oxygen uptake measured at the end of one hour. Except where otherwise stated, all the results given were calculated by the first method. In the early experiments the cats were killed by gassing with coal gas and cutting the neck vessels when the cats were unconscious. In the later experiments the cats were killed by giving an intraperitoneal injection of sodium amytal (for a supply of which we are indebted to Messrs. Eli Lilly & Co. Ltd.), and when the cats were anaesthetized the abdomen was opened and the aorta was incised. The results were the same by the two methods of killing.

The arteries of each foreleg of the cat were dissected immediately after death, dried on filter paper, weighed, and at once stored at −10° C. For the manometric experiments they were prepared as has been described for the nictitating membranes. Approximately 0.3 g. tissue was suspended in 4 ml. phosphate buffer, and 1.6 ml. of this suspension was placed in each manometer flask. Tyramine was used as the substrate and observations were made in the presence of KCN. In one experiment shown in Table V, performed nine days after denervation, the tissue was prepared as a homogenate instead of by alternate chopping and pounding ; as a result the O_2 uptake in the normal tissue was much greater than in the other experiments.

RESULTS

The nictitating membrane.—Table I gives details of eight experiments in which the normal right nictitating membranes were compared with the normal left membranes, and also of eight other experiments in which the denervated right membranes were compared with the normal left membranes, denervation having been performed nine to twelve days previously.

TABLE I

AMINE OXIDASE IN NICTITATING MEMBRANES EXPRESSED AS μl. O_2/g./hr.

Exp.	Normal left	Normal right	Denervated right	Days denervated	Right as % of left
41	147	168	—	—	114
42	126	133	—	—	105
43	200	223	—	—	112
44	69	81	—	—	117
45	64	60	—	—	94
46	102	143	—	—	140
47	72	85	—	—	118
48	95	114	—	—	120
3	125	—	111	9	88
4	152	—	65	9	42
32	273	—	122	9	45
5	114	—	34	10	30
6	115	—	66	10	57
24	118	—	68	10	58
25	32	—	31.5	10	98
10	116	—	52	12	44

The mean figure for the normal right membranes was 115 per cent of the normal left membranes, whereas the mean figure for the denervated right membranes was 58 per cent. The difference between these figures is highly significant ($P<0.01$), and the conclusion can be drawn that in the period of nine to twelve days after

denervation the amine oxidase in the membrane fell to not much more than half its initial value. The higher mean value for amine oxidase in normal right membranes than in normal left membranes may be a true finding, since the standard error of the value 115 was not more than 4.65. On the other hand, the normal controls were examined first of all, and it may be that there was some difference in the dissection of the nictitating membranes of the two eyes which was eliminated by practice.

Variation in fall in amine oxidase.—The figures in Table I for amine oxidase in denervated membranes show much variation. Two are as high as 98 and 88 per cent, while one is as low as 30 per cent. This variation at first made it seem unlikely that the fall in amine oxidase could account for the increased sensitivity of the membrane to the action of noradrenaline and adrenaline. We knew, however, that there are differences between cats in the response of the normally innervated nictitating membrane to noradrenaline. In some cats even large doses of noradrenaline produce almost no effect on the normal membrane, whereas in others smaller doses produce an easily measured response. We therefore decided to measure the degree of sensitization produced by denervation in order to see whether it was correlated with the change in the amount of amine oxidase.

Twenty-one cats were then prepared by removal of the right superior cervical ganglion, and after varying intervals each cat was anaesthetized, the cervical sympathetic chain was divided on the left side, and a spinal preparation was made. The nictitating membranes were threaded so that the contractions in them could be recorded. A series of injections of noradrenaline and of adrenaline was given to determine the ratio of the contractions in the two membranes. When sufficient observations had been made, the nictitating membranes were removed from the cat and were at once stored at $-15°$ C. until manometric observations could be carried out.

Results in three cats are shown in Fig. 1, from which it will be apparent that there was a great variation in the degree of sensitization produced by denervation. In Fig. 1,A the sensitization was extreme, since the injection of 7 μg. noradrenaline caused a scarcely perceptible movement of the normal nictitating membrane, but a large contraction of the denervated membrane. In Fig. 1,B there was no sensitization at all. Both the normal and the denervated membranes contracted to the same extent. Yet the cats used to obtain the records in Fig. 1,A and in Fig. 1,B were prepared in the same way by the same worker and the operation for removal of the superior cervical ganglion was performed on each cat 9 and 8 days respectively before the final experiment. Finally, in Fig. 1,C is shown a result intermediate between those in sections A and B of the same Figure ; in response to the injection of 7 μg. noradrenaline there was a small contraction of the normal nictitating membrane and a larger contraction of the denervated membrane.

Relation between sensitization and amine oxidase.—In the experiment on each cat we gave a series of injections of noradrenaline and a series of injections of adrenaline, and we measured the contractions of the membranes so produced. The result of each injection was expressed as the ratio of the contraction in the denervated membrane to that in the normal membrane (D/N ratio). The mean ratios for the different injections of noradrenaline, and for the different injections of adren-

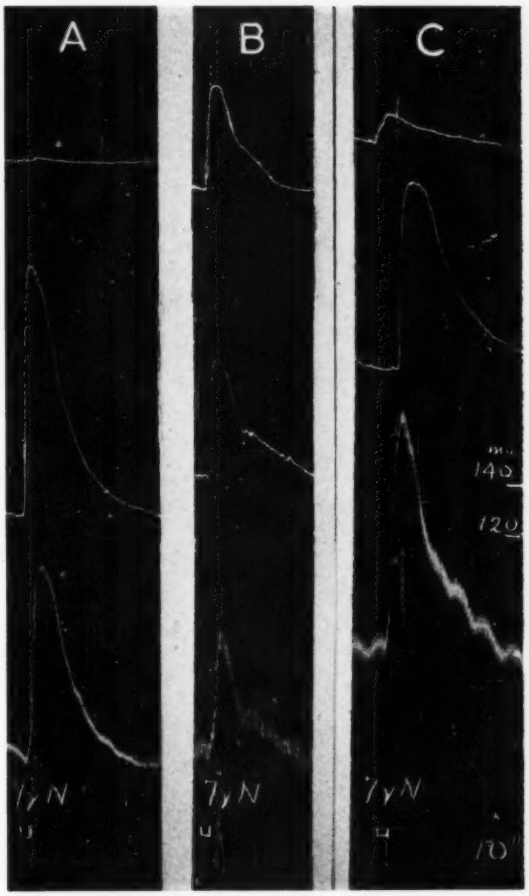

FIG. 1.—Responses to 7 μg. noradrenaline in 3 cats. Top record is normal nictitating membrane. Middle record is denervated membrane. Bottom record is arterial blood pressure. Note that the ratio of the contraction of the denervated to the normal membrane (D/N) is very high in A, is about unity in B, and has an intermediate value in C. The amine oxidase content of the membranes was determined, and the amount in the denervated membrane expressed as a percentage of that in the normal membrane.

	A	B	C
Mean D/N ratio	48.5	1.7	11.1
Amine oxidase %	43	95	66

aline, were calculated for each cat. The different ratios for the three experiments illustrated in Fig. 1 are set out in Table II. The range of doses used was from 5–10 μg. in all experiments. Although there was some variation in the ratios for noradrenaline and for adrenaline in each experiment, it will be seen from Table II that this variation was not great.

A determination of the amount of amine oxidase in the nictitating membranes of each cat was also made. In the first cat of Fig. 1, where denervation produced great sensitization, there was a large fall in amine oxidase in the denervated membrane to 43 per cent of that in the normal membrane. In the second cat of Fig. 1, where denervation failed to sensitize, there was no appreciable fall in amine oxidase, the figure obtained being 95 per cent. In the third cat, where denervation produced a moderate degree of sensitization, there was a fall in amine oxidase to 66 per cent of that in the normal membrane. Thus there was correspondence between the degree of sensitization and the fall in amine oxidase in the three cats.

Similar observations were made in seven other cats during the period 8–12 days after denervation and the results are given in Table III. In order to determine

TABLE II

Calculation of the D/N ratio, this being the mean figure for the ratio of the contractions in the denervated and normal membranes, from the results of the experiments illustrated in Fig. 1

	Exp. A		Exp. B		Exp. C	
	Dose μg.	D/N ratio	Dose μg.	D/N ratio	Dose μg.	D/N ratio
Noradrenaline ..	5	52	8	2.1	8	7.0
	7	52	6	1.4	4	12.5
	5	45	5	2.1	5	10.8
	6	45	6	1.5	6	14.3
			7	1.1	5	11.0
			10	2.3		
Mean ..	5.75	48.5	8.4	1.75	5.6	11.1
Adrenaline	7	4.5	10	0.7	10	1.6
	10	3.4	10	0.5	10	1.4
	10	4.0	10	0.8	12	1.5
	12	5.9	10	1.0	12	1.5
	10	7.25	10	1.0	10	1.5
Mean ..	10	5.0	10	0.82	10.4	1.5

TABLE III

Increased sensitivity to noradrenaline and adrenaline of denervated membrane, measured by the D/N ratio, and the percentage of amine oxidase in the denervated membrane

Exp.	Days denervated	μl. O_2/g./hr. in normal membrane	Amine oxidase percentage in denervated		D/N ratio		
					Noradrenaline		Adrenaline
A	9	113	43		48.5		5.0
B	8	89	95.		1.75		0.82
C	9	112	66		11.1		1.5
4	8	78.5	57		17.7		1.81
5	10	100	29		28.3		3.75
6	10	96	49	62.4	29.1	15.9	4.45
7	8	86	98		4.4		1.7
8	8	107	75		9.8		1.4
9	8	76	71		4.65		1.0
10	12	100	41.5		4.0		1.1
11	19	65	44		26.4		5.2
12	19	54	70		0.9		0.4
14	25	108	66		10.2		0.5
15	25	114	85		31		6.1
16	26	53	85		8.4		2.26
17	26	82	64	83.3	2.6	12.1	0.57
18	26	102	86		15.6		2.67
19	28	62	141		6.5		1.66
20	28	96	59		0.8		0.41
21	33	68	100		10		2.0
22	33	82	117		20.6		4.4

whether the percentage of amine oxidase in the denervated membrane was correlated with the D/N ratio the results for amine oxidase were plotted as abscissae in Fig. 2 against the logarithm of the D/N ratios ($\times 10$) for noradrenaline as ordinates and in Fig. 3 against the corresponding figures for adrenaline as ordinates. The values for amine oxidase were calculated from the extra oxygen uptake at the end of 1 hr. These values were rather more closely correlated with the nictitating membrane contractions than those calculated from the rate of oxygen uptake in the first period of 20–30 min.

We are indebted to Dr. D. J. Finney for Figs. 2 and 3 and for the following statements : " The correlation coefficients for log D/N ratio with amine oxidase were –0.709 for noradrenaline and –0.634 for adrenaline as compared with –0.632 for the 5 per cent significance level. The figures for the D/N ratios for noradrenaline were themselves closely correlated with the figures for the D/N ratios for adrenaline, the correlation coefficient being –0.919. The regression line of log D/N ratio for noradrenaline (Y_1) on amine oxidase percentage x was

$$Y_1 = 2.895 - 0.0143x$$

and the corresponding regression line for adrenaline (Z_1) was

$$Z_1 = 1.747 - 0.0076x$$

Significance of the correlation coefficients implies that these regressions (which are drawn in Fig. 2 and in Fig. 3 respectively) are significant."

These results can be criticized on the ground that the relative size of the contractions of the denervated and normal nictitating membranes depends on the dose,

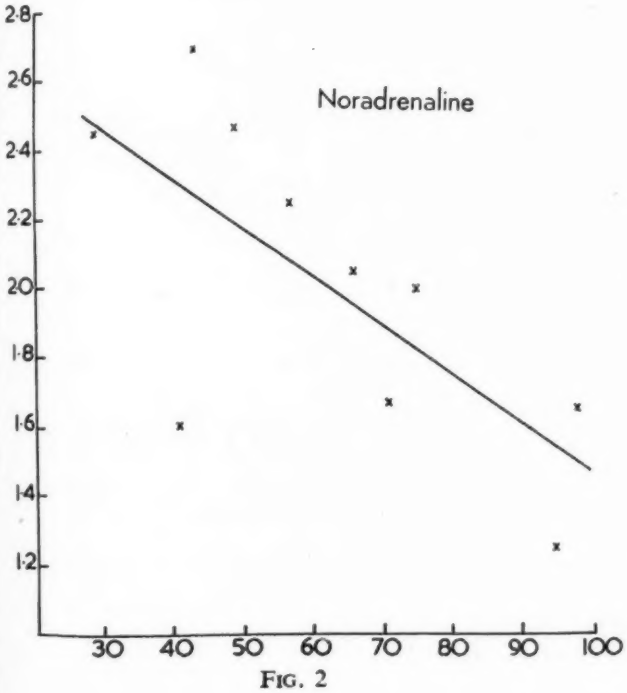

FIG. 2

FIG. 2.—Showing relation between logarithm of D/N ratio of nictitating membranes to noradrenaline (ordinate) and percentage of amine oxidase in denervated membrane (abscissa), in 10 cats examined between 8–12 days after denervation. The equation to the regression line is given in the text.

FIG. 3.—A similar relation to that in Fig. 2, but obtained by injecting adrenaline.

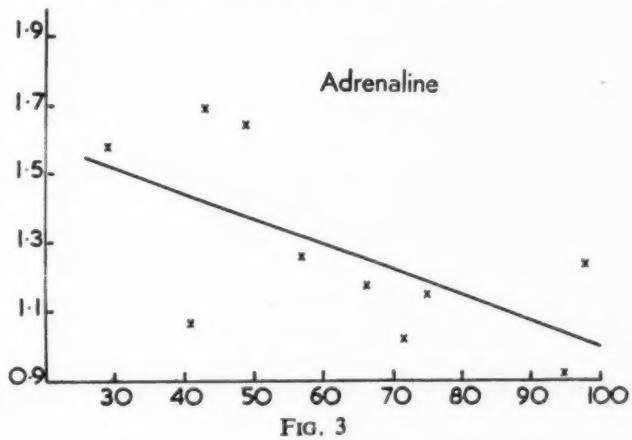

FIG. 3

the D/N ratio becoming smaller as the dose increases. However, the doses used in the different experiments were similar, ranging for noradrenaline from 4 to 10 μg., the mean being 7.2 μg., and ranging for adrenaline from 9 to 13 μg., except in Exp. 5, in which 3 doses of 20 μg. were used, thereby probably giving too low a ratio. So far as noradrenaline was concerned the D/N ratios varied greatly in different experiments, and errors in estimating each, due to difference in dosage, could not have changed them appreciably. So far as adrenaline was concerned the dose used was very nearly constant throughout. Since the normal membrane contracts fairly well in response to adrenaline, the ratios could be determined more accurately than for noradrenaline. Since, however, they did not greatly differ, the significant correlation of these ratios with the amine oxidase percentage in the denervated membrane was quite unexpected and gives strong support to the evidence.

Denervation for longer periods.—The effects of denervation so far described, the fall in amine oxidase correlated with the increased sensitivity of the membrane, were observed in the period 8–12 days after removing the ganglion. Further experiments were then carried out at longer intervals, from 19–33 days, and in these there was no correlation between the fall in amine oxidase and the increase in sensitivity. The results of these experiments are given in Table III, together with those which appear graphically in Fig. 2.

Table III shows that the mean amine oxidase percentage was higher in the experiments in which the period of denervation was longer. Whereas the mean percentage for cats denervated 8–12 days was 62.4, the mean percentage for cats denervated 19–33 days was 83.3. The sensitivity of the membrane to noradrenaline moved in accordance with this, the mean D/N ratio falling from 15.9 to 12.1. Thus if the deficiency of amine oxidase in the denervated membrane at 8–12 days was taken as 100, then the deficiency in the period 19–33 days was $\frac{62.4}{83.3} \times 100 = 75$ per cent of this deficiency ; similarly, if the supersensitivity of the denervated membrane to noradrenaline at 8–12 days was taken as 100, then in the period 19–33 days it fell to $\frac{12.1}{15.9} \times 100 = 76$ per cent. Thus the mean change in amine oxidase agreed closely with the mean change in sensitivity.

The rise in the amine oxidase at the longer period of denervation was a very surprising and wholly unexpected observation. In addition to the results in Table III, 18 other experiments had been carried out at different intervals after denervation in which the change in amine oxidase alone was measured. In each of these experiments two cats were used, so that we had information on the changes in amine oxidase in the denervated membrane in a total of 57 cats (including those in Table III). The results were arranged in groups according to the time after denervation at which they were obtained, and they appear in Fig. 4.

Fig. 4 shows that the amine oxidase percentage was lowest 10–12 days after denervation, and then steadily rose to the normal value which it reached about four or five weeks after the removal of the ganglion. From these results it was clear that denervation initiated two changes, a fall presumably due to the removal of nervous control reaching a low point after 10–12 days, and also a recovery process whereby the deficiency of amine oxidase was gradually made good perhaps

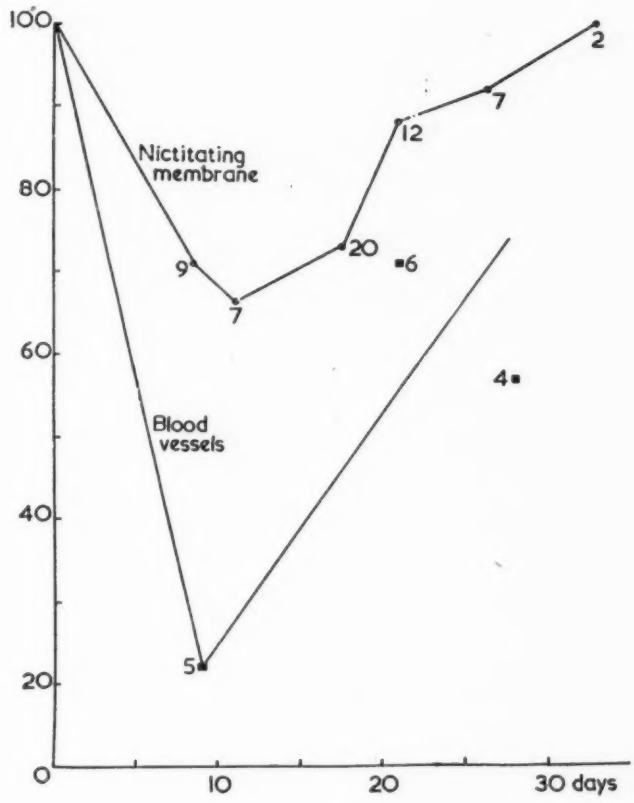

FIG. 4.—The percentage of amine oxidase in the denervated organ, compared with that in the normal organ, is recorded as ordinate, and the number of days after denervation is recorded as abscissa. The upper curve is drawn from observations on the nictitating membrane in which 39 manometric experiments were performed. The lower curve is drawn from observations on the foreleg arteries in which only 3 manometric experiments were performed. The figure beside each point is the number of cats used.

from some source outside the membrane. The failure to observe a correlation between the sensitivity of the membrane and the amine oxidase percentage in the later period thus received a possible explanation.

Effect of cocaine.—The evidence which has been given that the increased sensitivity of the denervated membrane to noradrenaline and adrenaline is caused by the decline in the amount of amine oxidase is supported by the effect of cocaine. Fröhlich and Loewi (1910) first showed that cocaine increased the action of adrenaline on the blood pressure ; later Rosenblueth and Cannon (1932) showed that it increased the action of adrenaline on the nictitating membrane. In 1940 Philpot demonstrated that cocaine inhibited the action of amine oxidase *in vitro.*

Cocaine hydrochloride was injected intramuscularly in 4 doses each of 2 mg. in different sites in the spinal cat. Two changes were often seen which are illustrated in Fig. 5. There was a rise of blood pressure, which was maintained, and there was an increase in the tone of the normally innervated nictitating membrane. Both these changes can be explained by inhibition of amine oxidase, since the denervated membrane did not increase in tone. We presume that in the normal membrane and in the blood vessels the existing tone represented the balance between the production of noradrenaline at the nerve endings and its destruction by amine oxidase. When cocaine was given and the enzyme was inhibited, the balance was altered in favour of increased tone.

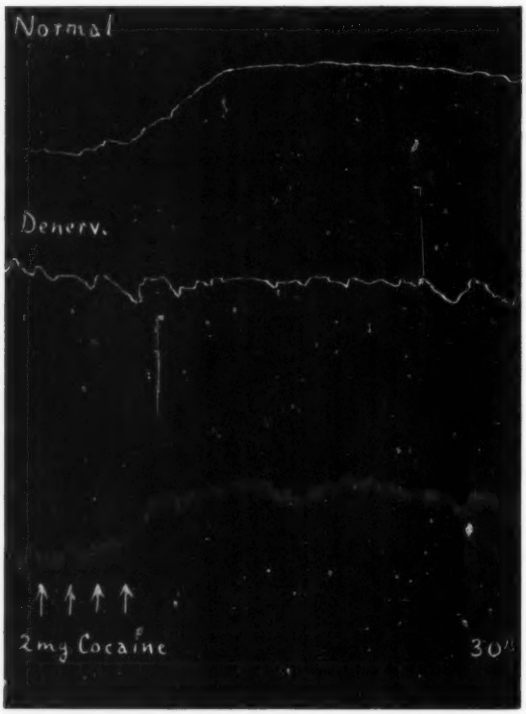

FIG. 5.—To show that the injection of cocaine in four doses of 2 mg. each into the muscles of a cat caused a rise of blood pressure (bottom record), a contraction of the normal membrane (top record), but no contraction of the denervated membrane (middle record).

The response of the normally innervated nictitating membrane to noradrenaline was greatly increased by cocaine, while the response of the denervated membrane was little affected, being increased occasionally. The ratio of the contraction of the denervated membrane to that of the normal membrane was reduced so as to be nearly equal to unity. In five experiments in which the contractions of the normal and denervated membranes were recorded, the mean D/N ratios for all injections of noradrenaline given before cocaine were consecutively 18.0, 3.0, 32, >50, and 1.0. After cocaine they became 1.4, 0.8, 1.3, 1.3 and 0.7, the mean figure being 1.1. Table IV shows the change produced by cocaine on the contractions caused by one dose of noradrenaline in each of these experiments.

TABLE IV

EFFECTS OF COCAINE ON CONTRACTIONS OF NORMAL AND DENERVATED NICTITATING MEMBRANES

Exp.	Dose: μg. Noradrenaline	Before cocaine			After cocaine		
		Contraction (mm.)		D/N	Contraction (mm.)		D/N
		Normal	Denerv.		Normal	Denerv.	
1	9	2	24	12	12	23	1.9
2	7	5	10	2	12	9	0.75
3	10	0	20	>20	11	15	1.36
4	7.5	0	28.5	>28.5	26.5	34	1.28
5	8	11	11	1			
5	15				24	17.5	0.73

The blood vessels.—The results for the blood vessels in a series of cats are shown in Table V. Three experiments were performed, each on a group of 4–6 cats in which the right foreleg arteries, not previously denervated, were compared

TABLE V

AMINE OXIDASE IN ARTERIES OF FORELEG OF CAT EXPRESSED AS μl. O_2/g./hr.

Number of cats	Normal left	Normal right	Denervated right	Days denervated	Right as % of left
6	360	370	—	—	103
4	365	315	—	—	86
5	202	213	—	—	105
5	1,380	—	395	9	22
6	375	—	265	21	71
4	272	—	154	28	57

with the left foreleg arteries. When the amine oxidase activity of the right leg vessels was expressed as a percentage of that in the left, the figures were 103, 86, and 105. In three other experiments in which the right foreleg was previously denervated, the figures were, after 9 days, 22 and, after 21 and 28 days, 71 and 57 respectively. Thus in the arteries as well as in the nictitating membrane the amine oxidase fell after denervation, the fall being greatest at 9 days and less after 3–4 weeks, suggesting that the amine oxidase was being restored. The results are shown in Fig. 4. We would emphasize, however, that these are results from three manometric experiments only.

The iris.—The results with the iris on the whole corresponded with the results on the nictitating membranes and the arteries. Two experiments in which the normal iris of one side was compared with the normal iris of the other, the number of cats used being six in the one experiment and five in the other, gave good agreement, as shown in Table VI. The amine oxidase in the denervated iris was lowest compared with the normal iris when the observations were made 7–14 days after denervation. The remaining observations were made from 15–26 days after removal of the ganglion and are consistent with a return towards the normal value in this period.

TABLE VI

AMINE OXIDASE IN IRIS OF CAT EXPRESSED AS μl. O_2/g./hr.

Number of cats	Normal left	Normal right	Denervated right	Days denervated	Right as % of left
6	162	165	—	—	102
5	205	195	—	—	95
4	451	—	100	7–14	22
5	336	—	256	15	76
6	352	—	306	16	87
6	214	—	235	20	110
7	241	—	230	21	95
6	192	—	157	21	82
3	197	—	136	26	69

Discussion

The evidence, that after degeneration of the sympathetic fibres in the nictitating membrane, in the arteries, and in the iris of the cat there is a fall in the amount of amine oxidase, indicates that there is a close relation in function between the nerves and this enzyme. The fall in the amount of enzyme in the nictitating membrane is greatest between 8 and 12 days after extirpation of the ganglion, but its extent varies greatly. The residual enzyme was observed to be as little as 29 per cent of that in the normal membrane or as much as 98 per cent, but whatever it was the fall in enzyme was correlated with the magnitude of the increased sensitivity of the denervated membrane to noradrenaline and also to adrenaline. This correlation affords strong evidence that when noradrenaline or adrenaline is injected the effect it exerts on the nictitating membrane is determined by the amount of amine oxidase present and not by the amount of any other enzyme. The further conclusion seems justified that noradrenaline liberated as the sympathetic transmitter is also destroyed by amine oxidase.

In the blood vessels of the cat's foreleg and in the iris of the cat's eye there was also a fall in amine oxidase after denervation which in the vessels was greatest at 9 days. These results suggest that amine oxidase destroys noradrenaline both in the blood vessels and in the iris. The conclusion is supported by the effect of cocaine, which is a substance known to inhibit amine oxidase. When injected into the spinal cat cocaine usually caused the blood pressure to rise and the tone of the innervated nictitating membrane to increase ; there was no change in the tone of the denervated membrane. Presumably there was a continuous release of noradrenaline at the nerve endings in the membrane and in the vessels, corresponding perhaps to the continuous release of acetylcholine at the motor endplate, described by Fatt and Katz (1950) and called by them " endplate noise." When the rate of destruction of this continuously released noradrenaline was reduced by the injection of cocaine, the tone in the nictitating membrane and the vessels was correspondingly raised.

The injection of cocaine likewise increased the response of the innervated nictitating membrane to noradrenaline so that it became practically the same as the response of the denervated membrane. This was true whether the difference in sensitivity of the two membranes was large or small. Such a change should occur if the increased sensitivity of the denervated structure is due to the fall in amine oxidase.

Four arguments against amine oxidase as the agent which destroys adrenaline or noradrenaline in the nictitating membrane require consideration. Bacq (1949) has pointed out that, when it was shown by Philpot (1940) that cocaine inhibits amine oxidase *in vitro,* she found that other local anaesthetics were equally strong or stronger inhibitors, but, he says, " there is no parallelism between the degree of enzyme inhibition and the sensitizing power." For example, cinchocaine (percaine or nupercaine) was the best inhibitor *in vitro,* yet it had only a slight potentiating action compared with that of cocaine (Bacq and Lefèbvre, 1934). If two substances act similarly *in vitro,* it is easily possible that they may act differently *in vivo,* since their action *in vivo* will depend on the rate of absorption when instilled into the eye, and will depend on both having a structure with the same adsorption and partition properties when injected intravenously during transit to the site and at the site itself (Albert, 1951).

Bacq (1949) further states that the view that cocaine causes sensitization by inhibition of amine oxidase does not explain why cocaine abolishes the action of tyramine instead of increasing it. There is now evidence which is fairly complete, and which will be discussed in another paper, that the action of tyramine in the body (excepting perhaps on the heart) is due to its attraction for amine oxidase, which destroys tyramine in preference to destroying other substrates. Tyramine has very little action on denervated structures (Burn and Tainter, 1931 ; Burn, 1932 ; Bülbring and Burn, 1938). Consequently when cocaine is first given and amine oxidase is inhibited, tyramine is without appreciable effect. Tyramine is thus similar in action to ephedrine ; both substances lead to the deviation of amine oxidase from its normal task ; tyramine is, however, destroyed and its action is of short duration, while ephedrine is not destroyed.

A third argument which has been put forward against the view that the sensitization of the nictitating membrane is due to the fall in amine oxidase is that the substance Corbasil (or Cobefrin) which has a $-CH_3$ group on the α carbon atom of the side chain, and which is not destroyed by amine oxidase, causes a greater contraction of the denervated membrane than of the normal membrane. It should be said in the first place that the difference in the sensitivity of the two membranes is far less for these substances than it is for noradrenaline. The maximum D/N ratio observed by Bülbring and Burn (1949) for Corbasil was 2.4, whereas for noradrenaline the ratio may be as great as 50. In the second place substances with a $-CH_3$ group on the α carbon atom, which inhibit the action of amine oxidase on adrenaline, must do so by combining with the enzyme, although they are not destroyed by it. When Corbasil reaches the nictitating membrane in the body, some of the molecules will therefore combine with the enzyme, and the remaining molecules will cause the membrane to contract. In the denervated membrane, where there is less enzyme, there will be more molecules free to cause the contraction. The contraction will therefore be greater.

Finally Rosenblueth (1932) has found that the denervated membrane is supersensitive to acetylcholine, pilocarpine, eserine, and histamine, and (Bacq and Rosenblueth, 1934) to calcium and potassium salts. Cannon and Rosenblueth (1949) therefore argue that "the supersensitivity cannot reasonably be attributed to a decrease in amine oxidase." Without discussing this argument in detail we would point out that it is unlikely that all these agents have their own specific receptors on the nictitating membrane ; it is more likely that they have some small power of attaching themselves to the receptors on which noradrenaline and adrenaline act, and therefore to the receptors on the enzyme as well. For this reason they will have a greater action on the denervated membrane, where there is less enzyme, just as Corbasil has. More work is certainly required on this point, but it is clear that the objection of Cannon and Rosenblueth is not unanswerable.

On the basis of our observations, that in the first 12 days after removal of the ganglion the increased sensitivity of the denervated membrane is correlated with a fall in amine oxidase, we consider that the supersensitivity of denervated structures (sometimes referred to as Cannon's law) is explained in the nictitating membrane by the fall in the enzyme.

Our results have shown that after reaching a low point about the 10th–12th day the amine oxidase in the denervated membrane slowly returns to the normal at about

4–5 weeks. This return was surprising, since the superior cervical ganglion was extirpated and there seemed to be no possibility of regeneration of the nerves. Although our evidence in denervated arteries is less complete, in them also we found the fall in amine oxidase greatest at 9 days and much less later. There are no other records of such a restoration of enzyme in course of time after denervation, but two other observations may be relevant. Keil and Root (1941) found that after extirpation of the ciliary ganglion the sensitivity of the cat's pupil was greatly increased in six days, and then in the next four weeks it steadily declined. Examination of the figures of v. Euler and Purkhold (1951) shows that the amount of noradrenaline in the spleen and the kidney fell very low 8–15 days after denervation, but was then partially restored after 3–4 months. In the latter experiments regeneration of the nerves might have occurred.

The restoration of amine oxidase in the nictitating membrane, and probably in the blood vessels, may be due to the transference of the enzyme from the liver. The restoration in the membrane was accompanied by a decline in the mean sensitivity to the action of noradrenaline, but there was no correlation in individual cats between the sensitivity and the amount of enzyme such as was observed at 8–12 days. For this reason the suggestion arose that the amine oxidase accumulating once more in the membrane might not always reach those points where it was effective in controlling the reaction of the membrane to noradrenaline. There were two other considerations in favour of the hypothesis that amine oxidase can be present, not only in the denervated but also in the normal membrane at two sites, namely, at points where it is effective and also at points where it is ineffective.

The first of these can be appreciated by examining Fig. 1, Exp. B. In this experiment there was practically no fall in the enzyme after denervation, and the contractions in the two membranes were almost the same. Both, however, were large. In experiments such as this, where the fall of enzyme after denervation was slight or absent, the sensitivity of the normal membrane to noradrenaline was always unusually great. The amount of amine oxidase in the normal membrane would therefore be expected to be unusually small. Actually the amount of enzyme was not unusually small, as Table III shows. There was, however, very little loss of enzyme on denervation. The inverse relation between the size of the response of the normal membrane and the extent of the fall in amine oxidase in the denervated membrane, well shown in the three parts of Fig. 1, are, however, explained on the hypothesis that the amine oxidase exists at (*a*) effective sites, and (*b*) ineffective sites, and that after denervation a fall occurs only in the amount present at the effective sites. In Fig. 1, Exp. B, there was little enzyme at effective sites and therefore the response of the normal nictitating membrane was large ; further, since the amount of enzyme at effective sites was small, the fall in amine oxidase on denervation was also small.

The action of cocaine can also be clarified on this hypothesis. Denervation in no experiment reduced the amine oxidase to zero, and therefore cocaine, by inhibiting the residue of amine oxidase, should have increased the response of the denervated membrane to noradrenaline. But this was not observed. Cocaine rarely increased the response of the denervated membrane ; its effect was to increase the response of the normal membrane until it was closely similar to that of the denervated membrane. This result is explained if it is supposed that the amine oxidase

still present is at an ineffective site, and that its inhibition by cocaine makes no difference to the response of the membrane to noradrenaline.

The hypothesis that amine oxidase exists at effective and ineffective sites can now be applied to the restoration of the enzyme in the denervated membrane. If we assume that the accumulating enzyme is deposited at both sites we then understand why there is a return of the sensitivity to noradrenaline towards normal, but no correlation in individual membranes between the sensitivity and the total amount of enzyme.

In conclusion it may be said that the evidence presented in this and an earlier paper (Burn and Robinson, 1951) supports the hypothesis of Burn and Hutcheon (1949) that the relative weakness of noradrenaline on the normally innervated nictitating membrane, iris, and blood vessels is explained by the presence of an enzyme which destroys noradrenaline more rapidly than it destroys adrenaline. In these organs we consider that amine oxidase plays the same role in destroying noradrenaline which cholinesterase plays in destroying the humoral transmitter of parasympathetic impulses.

SUMMARY

1. The nictitating membrane, the iris, and the blood vessels of the cat have been shown to contain amine oxidase. A study has been made of the effect of extirpating the ganglion, with consequent degeneration of the postganglionic fibres, upon the amount of amine oxidase present.

2. Denervation results in a fall in amine oxidase in all three tissues, the amount of enzyme in the denervated side being compared with the amount on the opposite side. The vessels of one foreleg were denervated by removing the stellate ganglion.

3. In the nictitating membrane the fall is very variable, and it is greatest at 8–10 days after denervation. The increase in the sensitivity to noradrenaline of the denervated membrane was measured, and at 8–10 days after denervation it was found to be significantly correlated with the fall in the amount of the enzyme. Similarly, the increase in the sensitivity to adrenaline was also measured, and it was also significantly correlated with the fall in the amount of the enzyme.

4. About ten days after denervation the amount of amine oxidase in the denervated membrane was found to rise again and by 33 days to be equal to the amount in the normal membrane. The course of the fall and rise was studied in a total of 57 cats.

5. In the blood vessels and in the iris also there appears to be an early fall in the amine oxidase, greatest at about 10 days, with a subsequent return towards normal.

6. The effect of cocaine on the nictitating membrane is consistent with the view that its action is due to an inhibition of amine oxidase.

7. The results indicate that amine oxidase at the postganglionic terminations in the nictitating membrane, the blood vessels, and the iris plays a similar part to cholinesterase at cholinergic nerve endings.

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THE NATURE OF CARDIAC SYMPATHIN IN THE DOG

BY

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Since the demonstration by Peart (1949) that stimulation of the splenic nerves of the cat releases a "sympathin" consisting predominantly of noradrenaline with a small admixture of adrenaline, similar results have been obtained for liver sympathin by Mann and West (1950) and for the substance released by stimulation of the hypogastric nerves by the same authors (1951).

Evidence on the nature of cardiac sympathin is available only for the frog's heart. Loewi (1936) demonstrated the actual release, on nervous stimulation, of a substance having the properties of adrenaline, and supporting evidence for its identity with adrenaline was obtained on extracts of heart muscle by Shaw (1938) and v. Euler (1946a). Cannon and Rosenblueth (1933) showed in the cat that stimulation of the cardio-accelerator strands from the right stellate ganglion caused the liberation into the blood mainly of a sympathin with excitor properties (sympathin E). Since that work, the identification of the sympathin released on stimulation of the nervi accelerantes has, as far as we know, not been attempted in the mammal. Mammalian heart extracts examined by v. Euler (1946b) were shown to contain a sympathin differing in its properties from pure adrenaline; this was later shown in cattle to consist of a mixture of noradrenaline with from 8 to 20 per cent adrenaline (Goodall, 1950).

The present paper is concerned with the nature of the sympathin released into the coronary blood of dogs on stimulation of the cardiac sympathetic nerves.

METHODS

Dogs were anaesthetized with ether followed by chloralose. Except for the first experiments, the splanchnic nerves were cut bilaterally below the diaphragm, the abdominal wound was closed, artificial respiration started, and the chest opened. The branches of the sympathetic leaving the stellate ganglia for the heart were traced on both sides and a ligature placed around the nerves. The pericardium was opened, both vagi cut, heparin (600–650 u./kg.) injected intravenously, and a Morawitz cannula introduced into the coronary sinus. The cannula was coated with a thin layer of hard paraffin.

Except for the periods when blood samples were being collected, the coronary blood was returned through a by-pass into a jugular vein. The sympathetic nerves were stimulated by alternating current from a Ritchie–Sneath stimulator (Ritchie, 1944), 7.5–10 V. and shielded platinum electrodes being used. Stimulation was started 5 sec. before the

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collection of a "stimulation sample" of blood and continued during the whole period of collection. Samples of pulmonary venous blood were taken from the right atrium and of arterial blood from the carotid or femoral artery, and collected sometimes during the stimulation and sometimes during the control periods. The blood was drained into ice-cooled centrifuge tubes and the plasma extracted. The extracts were chromatographed on paper, either by the procedure of Crawford and Outschoorn (1950) or by its modification which employs HCl-phenol instead of SO_2-phenol (see Vogt, 1952, for details concerning the preparation of the extracts and the chromatographic procedure). The eluates were tested for noradrenaline on the blood pressure of the atropinized hexamethonium-treated rat (Crawford and Outschoorn, 1950; Outschoorn, 1952) and for adrenaline occasionally on the rat's blood pressure but usually on the rat's uterus (Gaddum, Peart, and Vogt, 1949). Assays of native plasma on the rat's uterus proved impracticable owing to a high concentration of interfering substances in the coronary blood. The final eluates were concentrated so as to contain in 0.5 ml. the amines from 5 ml. plasma. The yield of recovery experiments and the precautions to be taken to check for substances in the eluates which may interfere with the assays are described in the papers to which reference has been made.

RESULTS

During stimulation of the two nervi accelerantes, the heart rate became faster and there was an increase in the coronary flow and in the force of the beat. The acceleration was more obvious on stimulating the right nerve. It did not reach its maximum before a latent period of some seconds. On stimulating the left nerve acceleration was often absent, but an inotropic effect could always be seen.

In three preliminary experiments, in which the splanchnic nerves were left intact, all samples of coronary plasma contained fairly high concentrations of adrenaline (up to 22 mμg./ml.), and on stimulation of the accelerator nerves these concentrations sometimes increased even further. Since at least part of this adrenaline seemed to be of medullary origin, the splanchnic nerves were cut in the remaining experiments, whereafter the highest concentration of adrenaline which could ever be ascertained in any sample was 1.5 mμg./ml.

Noradrenaline.—In all experiments, in which splanchnotomy had been performed, it was possible to demonstrate the release of noradrenaline on stimulation of the nervi accelerantes (see Tables I and II). The amounts ranged from 50 to over 700 mμg./min. The output per min. was approximately doubled by stimulating the nerves on both sides instead of on the right side only (Exp. 10).

Adrenaline.—In Exps. 4–6 the assay of adrenaline was attempted on the native plasma (using the rat's uterus) and on the concentrated eluates (using the rat's blood pressure). Neither of these tests proved sensitive enough to detect any adrenaline. By determining the threshold of the assay preparation, it was possible to establish that no more than one-third of the amines released might be adrenaline (compare the released noradrenaline recorded in the last column of Table I with the threshold for adrenaline listed in columns 5 and 6 and representing the maximum amount of adrenaline which might have been released on stimulation).

More information was obtained in Exps. 7–10, in which, after chromatography, the concentrated eluates were tested on the rat's uterus. The results are shown in Table II. Only in Exps. 9 and 10 were there traces of adrenaline (1–1.5 mμg./ml.) found in the coronary blood, but their occurrence was not cor-

TABLE I

ESTIMATION OF ADRENALINE AND NORADRENALINE IN PLASMA OBTAINED FROM THE CORONARY SINUS

Exp. No.	Nature of sample	Duration of collection (sec.)	Volume of plasma (ml.)	Output per min. (in mμg.)			
				Adrenaline*		Noradrenaline	
				Native plasma, rat's uterus	Eluates, rat's B.P.	Eluates, rat's B.P.	Increase during stimulation
4	Control	25	12.5	<300	<375	<186	
	Stimulation	28	21.0	<450	<562	279	>93
	Stimulation	25	16.0	<384	<480	960	>719
	Control	25	16.2	<389	<486	<241	
5	Control	30	7.0	<70	<175	—	
	Stimulation	30	6.0	<60	<150	—	
	Stimulation	30	7.0	<70	<175	175	>88
	Control	30	7.0	<70	<175	<87	
	Stimulation	30	4.0	<40	<144	144	>80
	Control	36	6.2	<52	<129	<64	
6	Control	30	8.0	S	<200	80	
	Stimulation	30	11.0	S	<275	136	56
	Stimulation	30	11.0	S	<275	550	>520
	Control	30	6.0	S	<150	<30	

S = stimulating effect on the rat's uterus by interfering substances.

* The figures in the columns headed "Adrenaline" represent the threshold amounts detectable in each instance by the particular preparation and thus the maximum amount of that substance which might have been released into the coronary blood.

TABLE II

ADRENALINE AND NORADRENALINE IN PLASMA OBTAINED FROM THE CORONARY SINUS

(Estimation after chromatographic separation)

Exp. No.	Nature of sample	Duration of collection (sec.)	Volume of plasma (ml.)	Output per min. (in mμg.)				Percentage methylation
				Total		Change during stimulation		
				Adrenaline	Noradrenaline	Adrenaline	Noradrenaline	
7	Control	35	13.3	<22.6	<141			
	Stimulation	60	10.2	<10.2	63	<10.2		
	Stimulation	60	8.0	—	100	—		
8	Control	60	12.0	<24	30			
	Stimulation	30	10.0	<40	266	<40	236	<15.6
	Stimulation	30	5.0	<20	500	<20	470	<4.2
	Stimulation	60	9.2	—	(560†)	—	—	
9	Control	45	9.0	12–18	30			
	Stimulation	45	8.0	<5.3	80	−10	50	(0)
	Stimulation	45	10.0	<26.7	133	<11.7	103	<10.2
10	Control	40	9.0	16.9	<8			
	Stimulation (R. nerve)	25	10.0	36.0	151	19.1	>143	<11.8
	Stimulation (Both nerves)	25	10.0	24.0	240	7.1	>232	<3.0
	Control	40	10.0	15.0	<38			
	Stimulation (Both nerves)	25	7.0	16.8	336	1.8	>298	<0.6

† Estimation in native plasma.

related with the activity of the nervi accelerantes, since they were found both before and during stimulation of the nerves and were also present in the arterial blood. Once, in fact, the quantity of adrenaline in the coronary blood became less during stimulation. The origin of these small amounts was obviously extracardiac and probably the result of direct stimulation of the adrenal medulla by anoxia due to low blood pressure. One possible exception was the finding of a release of 19 mμg./min. on stimulating the right accelerator nerve in Exp. 10. Even this figure, however, is based on a difference in adrenaline concentrations between the sample (1.5 mμg./ml.) and its control (1.25 mμg./ml.) which cannot be considered significant.

In the other experiments, no adrenaline was found, and the Table gives the upper limits for a possible output of this substance. From these figures the maximum possible percentage methylation of the released sympathin was calculated. The figures necessarily vary with the sensitivity of the assaying organ and the amount of noradrenaline produced, and range from 0–16 per cent. It is evident that, if any adrenaline is released on stimulation of the accelerator nerves, its quantity does not exceed a few per cent of the total amines.

When samples of pulmonary venous blood or of arterial blood were tested, the pulmonary venous plasma (collected during control periods) had no detectable adrenaline (<2 mμg./ml.), but, in Exps. 9 and 10, the arterial plasma contained a small quantity of adrenaline which was the same whether the plasma was collected during control periods or during nerve stimulation. Whenever the quantity of noradrenaline released into the coronary blood was high, there was, however, some noradrenaline to be found in those samples of arterial blood which were collected during stimulation of the cardiac nerves. Table III shows this phenomenon in Exp. 9.

TABLE III

Comparison of amine concentrations in plasma obtained simultaneously from the femoral artery and from the coronary sinus (Exp. 9). Estimation after chromatographic separation

Nature of sample	Concentration of amines (mμg./ml.)			
	Coronary plasma		Arterial plasma	
	Adrenaline	Noradrenaline	Adrenaline	Noradrenaline
Control	1.0–1.5	2.5	1.0–1.5	<2.5
Stimulation	<0.5	7.5	1.0–1.5	<2.5
Stimulation	<2.0	10.0	<2.0	2.5

Noradrenaline became detectable in the third sample of arterial plasma, when the concentration in the coronary plasma had reached 10 mμg./ml. (The samples of coronary blood were taken over a period of 45 seconds and an equal volume of arterial blood was collected in 10–15 seconds as soon as the accelerator effect was fully developed.

Discussion

The experiments show that stimulation of the nervi accelerantes in the dog causes the liberation of noradrenaline into the coronary blood. In no instance was there

evidence for the release of adrenaline on nerve stimulation, so that the methylated product is either not released at all or produced in amounts lying below the thresholds of the assays. In favourable experiments, this threshold was sufficiently low to exclude a percentage methylation above 3 per cent of the noradrenaline released. No attempt was made at identifying any other compounds which might be released. It is of interest that, of the two amines, *l*-noradrenaline is the more potent dilator of the coronaries (Smith and Coxe, 1950), that it has a greater inotropic action (Garb, 1950), and that, in the " fully " atropinized dog (0.4–0.6 mg./kg. of atropine sulphate), it is also the more potent accelerator of the heart (Lockett, 1950). Statements to the contrary regarding the effect on the heart rate are only in apparent contradiction to this, as the dose of atropine required to abolish the reflex slowing of the heart after a dose of noradrenaline is greater than that required to abolish the similar slowing produced by adrenaline. Thus the experiment reported by Pickford and Watt (1951), in which 0.13 mg. atropine sulphate per kg. did not prevent a slowing of the heart rate in a dog, is in good agreement with Lockett's observation that, in one animal, noradrenaline slowed the heart after 0.2 mg. atropine sulphate per kg., but accelerated it after 0.4 mg. per kg. The frog's heart, on the other hand, not only appears to use adrenaline as the sympathetic transmitter, but also to be more sensitive to the methylated product (West, 1947 ; Gaddum *et al.*, 1949).

The amounts of noradrenaline released on electrical stimulation of the cardiac nerves were occasionally sufficient to pass from the coronary sinus through the lungs and produce in the peripheral blood a detectable rise in circulating " sympathin."

The percentage methylation of extracts of cattle heart was reported by Goodall (1950) to amount to 8–20 per cent. Similar figures have been reported (Euler, 1951) for the sheep, and probably will also hold for the dog. Since no evidence for the release of adrenaline was obtained on stimulation of the nervi accelerantes, other sources of this amine may have to be considered, for instance, chromaffine tissue in the heart. The existence of such tissue was suggested by Hoffman, Hoffman, Middleton, and Talesnik (1945), who reported the release of an adrenaline-like substance when they injected acetylcholine or nicotine into atropinized perfused mammalian hearts. More evidence is, however, required to confirm that the substance released in this way does consist mainly of adrenaline.

Summary

Stimulation of the nervi accelerantes in the dog causes the release into the coronary blood of noradrenaline. Adrenaline is not liberated in detectable amounts by stimulation of the heart nerves. If any of the cardiac sympathin is adrenaline, its amount does not exceed 3 per cent of the noradrenaline released.

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THE SECRETION OF THE DENERVATED ADRENAL MEDULLA OF THE CAT

BY

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In 1917 Stewart and Rogoff tried to determine whether the chronically denervated adrenal medulla shows any secretory activity. They came to the conclusion that "no epinephrine is normally released except through nerves." In view of the recent development of sensitive methods for the detection of adrenaline and noradrenaline, and in view of the possible role played by noradrenaline in the maintenance of vascular tone, the problem was reinvestigated.

METHODS

Operations.—Ten cats were used. In a preliminary aseptic operation under ether, the larger and the lesser splanchnic nerves were cut bilaterally where they emerge from the diaphragm. In addition, both lumbar sympathetic chains were excised, from the first ganglion down to the third or fourth. The removal of the sympathetic chains ensures the section of any fibres which may enter the adrenal without first joining the splanchnic nerves (Elliott, 1913 ; McFarland and Davenport, 1941). Recovery from the operation was uneventful, except for an occasional transient diarrhoea. Between 17 and 56 days after the operation, the cats were anaesthetized with chloralose and adrenal blood collected after injection of heparin. In most instances, the blood was obtained from a cannula introduced into the lower end of the inferior vena cava after preliminary evisceration, bilateral nephrectomy, ligation of the aorta at the level of the renal arteries, and of the adreno-lumbar veins at the lateral border of the adrenals. The vena cava was occluded above the entry of these veins by a ligature which had frequently to be placed inside the liver tissue so as not to obstruct the flow from the right adrenal. For the purpose of arterial injections of KCl, a cannula was tied into the stump of the coeliac artery. The cannula was connected by a short piece of rubber tubing to the butt of a syringe needle. Slow infusions were made by a syringe, the contents of which could be delivered gradually by pushing its plunger with the help of a screw turning in a metal frame which was attached to the barrel. In a few animals the left adreno-lumbar vein entered the renal vein. In these cats, provided infusion into the coeliac artery was not intended, evisceration, right nephrectomy, and ligation of aorta and vena cava could be dispensed with. After removal of the left kidney, blood was obtained from the left adrenal by tying a cannula into the distal end of the left renal vein and occluding its central end.

Estimations of sympathomimetic amines.—All blood samples were collected in ice-cooled tubes, centrifuged without delay, and small portions of the native plasma tested for adrenaline on the rat's uterus on the same day. The remaining plasma was extracted, and the amines in the extracts separated by paper chromatography and eluted from the paper. The eluates were evaporated to dryness and the residue taken up in a

volume of water equal to one-tenth of the original volume of plasma. These solutions were assayed on the rat's uterus or on the rat's blood pressure (see below) as soon as possible; they were neutralized with solid $NaHCO_3$ immediately before use. Most assays were carried out on the day after the operation. If some of the tests had to be postponed to the following day, the evaporated eluates were stored in the refrigerator in the dry state and not dissolved till shortly before their assay.

Preparation of the extracts.—Plasma (5 ml.) was added to ethanol (30 ml.) containing 0.1 per cent (v/v) conc. HCl. After chilling for not less than half an hour, the mixture was centrifuged, the precipitate washed with more acid ethanol, centrifuged, and the combined supernatants evaporated to dryness *in vacuo* (bath temperature 45–50° C.). By means of small quantities of NaCl-saturated ethanol (total volume 6 ml.) the residue was transferred into a 15-ml. centrifuge tube, chilled, spun, the deposit washed with 1 ml. NaCl-ethanol, spun, and the combined supernatants evaporated to dryness as before. With the help of four 0.5 ml. portions of a mixture of equal parts of acetone and ethanol, the residue was transferred into a 15-ml. centrifuge tube, the solvent in the tube concentrated *in vacuo* to less than 1 ml., the tube chilled, centrifuged, and the supernatant applied to filter-paper for chromatography. The residue was washed with 0.25 ml. of the acetone-ethanol mixture, centrifuged, and the supernatant also applied to the paper.

The object of using NaCl-saturated alcohol for the second extraction was to reduce the amount of potassium salts in the solution (Barsoum and Gaddum, 1935), and that of using acetone-alcohol in the final extraction to lessen the amounts of inorganic constituents and of acetone-insoluble organic compounds applied to the paper.

Extracts of adrenal gland were prepared by grinding up one gland in 10 ml. 0.15 N-HCl, centrifuging, precipitating 1 ml. of the supernatant with 7 ml. ethanol, discarding any precipitate, and treating this alcoholic extract like the supernatant of a plasma extract in NaCl-ethanol. Assays were done only after chromatographic separation of the amines.

Chromatography.—In principle, the methods described by Crawford and Outschoorn (1950) were used, but three points of difference in the present procedure call for mention.

(1) The solvent employed for developing the chromatograms was a mixture of phenol with 0.1 N-HCl as used by Crawford (unpublished). Of different concentrations tried, 15 per cent (v/w) 0.1 N-HCl in phenol gave optimal separation without causing the occurrence of patches of waterlogging of the paper, as larger amounts of acid frequently did. The jars were filled with CO_2 or N_2 instead of SO_2.

(2) In the absence of SO_2 losses occurred unless the filter-paper was washed before use with 0.01 N-HCl, a procedure found by Crawford (unpublished) to be helpful in the fluorimetric estimation of noradrenaline.

(3) Chromatography was carried out at a temperature of 25–26° C., in order so to accelerate the flow that the process of separation was completed in 14–16 hours (overnight).

Assays.—The adrenaline, unless present in large amounts, was assayed on the rat's uterus stimulated by carbachol (Gaddum and Lembeck, 1949). Since this preparation is, on the average, 150 times more sensitive to adrenaline than to noradrenaline, interference with the estimation of adrenaline by the simultaneous presence of noradrenaline is usually negligible (Gaddum, Peart, and Vogt, 1949). Thus it was possible to assay both native plasma, which contained the two substances, and eluates of paper strips containing adrenaline only. When plasma was tested, difficulties were frequently encountered owing to the fact that certain samples, even when lacking any stimulating effect of their own on the uterus, were found to "mask" an appreciable quantity of added

adrenaline. Matching had therefore to be done with solutions of adrenaline in adrenaline-free plasma (i.e. arterial plasma, since all animals used had denervated adrenals) and not with adrenaline in saline. Even so, the results from a plasma which "masks" must be considered as somewhat unreliable, since the arterial blood need not contain the same amount of interfering substances as the adrenal effluent. Precautions were also necessary when eluates were being tested. The phosphates and traces of other chemicals which are present in the eluates may affect the isolated uterus and mimic or mask the presence of traces of adrenaline. Thus the volume of eluate added to the uterus bath has to be kept below the threshold of such interfering substances. The threshold is established by preparing an eluate from a strip of paper not containing any adrenaline. It was usually well above the quantity of eluate required for an assay. In cases of doubt, the neutralized solution was heated for 5 min. in a boiling waterbath, so that any adrenaline was destroyed, and the responses to the heated solution were examined without and with added adrenaline.

The assay of samples of adrenal blood collected during stimulation of the gland by KCl offered no difficulties. The adrenaline was estimated in the native plasma on the rat's uterus and in the eluate on the rat's blood pressure. The use of native plasma caused no errors, since the concentration of adrenaline was so high that the plasma could be diluted sufficiently to eliminate the action of masking substances. There was good agreement between the two methods.

Noradrenaline was only assayed after chromatographic separation from adrenaline, the assay of mixtures by parallel tests on different organs having been shown by Gaddum and Lembeck (1949) to involve very large errors. Noradrenaline was estimated by the rise in blood pressure produced in the rat under urethane anaesthesia, as described by Crawford and Outschoorn (1950). One modification of their procedure was the intravenous administration to the rat (Outschoorn, unpublished) of hexamethonium hydrobromide (5–10 mg./kg.). The threshold for noradrenaline is greatly lowered by hexamethonium, 1 mμg. noradrenaline being usually detectable at the beginning of the experiment. The sensitivity, however, gradually declines, and can only be partially restored by giving more hexamethonium.

Matching.—In the biological assays, matching was done by "bracketing" the unknown sample with two doses of standard differing by a factor of 2; thus if a result is listed as 7.5 mμg., it was found to be smaller than 10 and greater than 5 mμg. Usually the discrimination of the biological preparation was not sufficient to narrow down the range of the result any further.

Recovery.—Recovery experiments, in which 1 μg. adrenaline and 1 μg. noradrenaline were added to 5 ml. arterial plasma (taken from cats with denervated adrenals), showed the recovery to be rarely less than 75 per cent and sometimes more. The recovery of the two amines in a particular experiment did not differ significantly, so that the estimated percentage methylation should have been unaffected by occasional losses. When losses occurred they were always in the extraction and not in the chromatographic procedure, which allowed, within the limits of the error of the assay, full recovery of amounts as small as 5 mμg. adrenaline.

RESULTS

Resting secretion per minute.—Small quantities of sympathomimetic amines were found in all twelve samples of adrenal plasma tested. The mean secretion of both glands per min. was 21 mμg. adrenaline and 34 mμg. noradrenaline with a range shown in Table I. These outputs corresponded to an average concentration in the plasma of 1.4×10^{-8} adrenaline and 2.7×10^{-8} noradrenaline.

TABLE I

ADRENALINE AND NORADRENALINE IN PLASMA FROM DENERVATED ADRENALS

No. of cat	No. of sample	Post-operative interval (days)	Adrenaline (mμg./min.) assayed on		Noradrenaline (mμg./min.)	Blood flow (ml./min.)	Body weight (kg.)
			Uterus	Blood pressure			
1		52	5[1]		<48		5.0
2	1	56	46[1]	46	73	1.83	4.0
	2		46[1]	42	91	0.91	
3*	1	42	13[1]	15	43	0.86	3.6
	2		32[1]	40	80	0.32	
4	1	19	5[1]		6	1.0	3.5
	2		*1,500*[1]	*2,000*	*2,000*	*0.5*	
5	1	18	15[1]		20	1.25	3.3
	2		*3,500*[1]	*1,750*	*660*	*0.87*	
6	1	17	7[1]		19	0.57	2.5
	2		*2,500*[1]	*3,300*	*1,600*	*0.41*	
7*	1	23	10[2]		8	1.3	3.0
	2			*220*	*290*	*0.58*	
8*	1	26	48[2]		24?	4.8	2.8
	2		*2,600*[1]	*2,000*	*1,440*	*6.4*	
9*	1	28	28[2]		6	2.2	2.6
	2		*2,200*[1]	*2,600*	*630*	*0.7*	
10*	1	30	25[2]		8	1.18	1.8
	2		*46*†[1]	*110*	*70*	*0.92*	

* Indicates that blood was obtained from one gland only. In order to make the results directly comparable, the figures in columns 4–7 are doubled. [1] Native plasma tested. [2] Eluate tested. † Severe masking. *Italics:* during infusion of KCl.
Note.—In assays giving figures with more than one digit, the second digit is only approximate. Figures like 91 and 73 mμg /min. result from the conversion of observed concentrations into secretion per min.

In any one experiment the secretion per minute was steady, the concentration rising as the blood flow diminished. The only exception was Exp. 3, in which the blood pressure and the blood flow through the gland were intentionally reduced by severe haemorrhage between collection of the two samples of adrenal blood. Under the conditions of anoxia prevailing when the second sample was obtained the production of amines per minute by the glands had risen to twice its initial value.

Effect of KCl.—Arterial injection of KCl greatly accelerated the rate of secretion and the total amount of amines released per minute was usually of the order of 4,000 mμg.

Percentage methylation.—On the average, the percentage methylation (Table II) in the plasma collected from the resting glands was lower than the mean figure of 59 per cent reported by Bülbring and Burn (1949) for nine normal cats during a first electrical stimulation of the splanchnic nerves. The two sets of figures, however, have almost the same range, and in two denervated glands (cats 8 and 9) the percentage methylation was high even by normal standards.

Comparison of the figures obtained from the same cat under different conditions shows a striking similarity between percentage methylation in the blood collected at rest, in that obtained during stimulation of the medulla by KCl, and in the gland itself (cats 7–9). In cats 5, 6, and 10, on the other hand, methylation was found to be higher during stimulation by KCl than when the gland was at rest ; as

TABLE II

Cat No.	Sample No.	Percentage methylation of sympathomimetic amines		
		Adrenal plasma		Right adrenal gland
		At rest	During KCl infusion	
2	1	39		
	2	34		
3	1	25		
	2	31		
4		45	47	
5		43	80	
6		29	64	
7		56	43	38
8		66	62	69
9		82	79	83
10		23	53	
Mean of Nos. 4–10		49	61	
Mean of all determinations ..		43		

a result of this the mean percentage methylation in the secretion produced by chemical stimulation of the denervated gland does not differ from the value found for nervous stimulation of the normal gland.

Within the experimental period covered (up to 56 days), there was no correlation between the quantity or composition of medullary secretion and the interval between denervation and experiment.

Discussion

The experiments by Meier and Bein (1948), in which normal vasomotor responses were restored in the acutely adrenalectomized animal by infusing small doses of noradrenaline intravenously, suggest that a small quantity of this substance is continuously secreted by the adrenal medulla. The present experiments, carried out on denervated adrenals in which the nerves had been allowed time to degenerate, show that, in complete absence of nervous stimuli, blood leaving the adrenal medulla invariably contains noradrenaline, but also some adrenaline. The percentage methylation in this secretion tends to be low, but figures above 50 per cent occur, and the range covered is the same as that reported in the literature for normal cats.

Injection of KCl into such glands did not affect the percentage methylation except for three cats, in which it appeared to raise it. Since, however, the estimation of the percentage methylation in the resting samples, in which the concentrations encountered are often below 10^{-8}, is subject to large errors, these few observations can no more than suggest that injection of KCl may be able to increase the low percentage methylation of a denervated gland. The good agreement in percentage methylation between a gland excised at the beginning of an experiment and the blood leaving the other gland rather points to the degree of methylation present in the medullary tissue, and not the type of stimulus employed, determining the composition of the secretion. Further support for this view can be seen in the fact that

the average percentage methylation in the effluent of denervated glands stimulated by KCl is the same as that reported in the literature for normal glands secreting as a result of brief splanchnic stimulation.

Anoxia increases the secretion of the denervated adrenal medulla, though it is unable to accelerate it to an extent comparable with that occurring in the innervated gland. This observation is in good agreement with the observation by Bülbring, Burn, and Elío (1948) on the isolated adrenal of the dog.

The difference between the conclusions to be drawn from this work and the views held by Stewart and Rogoff (1917) are probably due to the recent improvement in experimental methods for the identification of minute quantities of sympathomimetic amines. The alternative explanation is that a few fibres escaped destruction in the operations carried out in the present experiments and that they might be the cause of the slight " resting " secretion ; this is not a very likely explanation, because there is no anatomical difficulty in tracing the splanchnic nerves and the sympathetic chains in a cat.

Summary

1. In ten cats, the nerves to the adrenal glands were cut and allowed to degenerate. Adrenal blood collected from these animals contained both adrenaline and noradrenaline, the mean secretion per min. amounting to 21 mμg. adrenaline and 34 mμg. noradrenaline. Though the percentage methylation tended to be low, its range covered that described for adrenal blood of normal cats in which the splanchnic nerves were being stimulated.

2. Arterial injection of KCl released sympathomimetic amines in the same proportion as stimulation of the splanchnic nerves does in the normal cat.

3. In spite of great individual variations, in any one animal the percentage methylation of the secretion at rest, of the secretion during injection of KCl, and of the stores in the medulla is usually about the same.

4. The observations under (2) and (3) suggest that it is the composition of the stores and not the type of stimulus at work which determines the proportion in which the adrenal medulla releases its two amines.

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It is a pleasure to thank Mrs. S. K. Pride for her skilful assistance in these experiments.

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THE PHARMACOLOGY OF BENZOYLCHOLINE

BY

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During a study of drugs possessing curare-like properties, Bovet and his co-workers (1949) noted that 50 mg./kg. of benzoylcholine caused head-drop in rabbits. It produced hypotension in dogs in doses of 0.1 mg./kg., and hypertension in higher doses. In addition, it caused contraction of the rabbit ileum and rectus abdominis muscle of the frog, and increased salivary secretion in dogs. Earlier, Carr and Bell (1947) found that benzoylcholine was much less active than acetylcholine in causing hypotension in dogs, and did not cause miosis in the rabbit's cornea. From these results it was concluded that benzoylcholine had strong nicotine-like properties and weak muscarine-like properties.

Because it contains a benzoic ester grouping, like many local anaesthetics, we first determined whether benzoylcholine possessed local anaesthetic properties. Secondly, we compared the actions of benzoylcholine and acetylcholine, and also the interactions between the two, with particular reference to the effect of benzoylcholine on heart muscle and the gut.

METHODS

The tests for local anaesthetic activity were carried out (*a*) on guinea-pig's skin (Bülbring and Wajda, 1945) and (*b*) on rabbit's cornea. For experiments on striated muscle, the isolated rectus abdominis of the frog (bath volume 2 ml.), the isolated phrenic nerve-diaphragm of the rat (bath volume 50 ml.), and the sciatic-gastrocnemius preparation of the cat under chloralose anaesthesia were used. Intravenous injections into week-old chicks (Buttle and Zaimis, 1949) were carried out in order to differentiate between true curare-like properties (flaccid paralysis) and decamethonium-like actions (spastic paralysis).

Pieces of rabbit and guinea-pig gut and guinea-pig uterus were suspended in an isolated organ bath (volume 15 ml.) containing Tyrode's solution at 37° C. For studying the peristaltic reflex, a large bath (volume 50 ml.) was used. Isolated tracheal chain preparations of the cat and the rabbit (Castillo and de Beer, 1947) were also used in a 15 ml. bath.

Cats anaesthetized with chloralose (60 mg./kg.) were used for studying effects on the arterial blood pressure recorded from the carotid artery. Rabbit and cat auricles were suspended in a bath of Locke's solution (15 ml.) at 30° C.; whole hearts were perfused by the Langendorff technique. Straub frog hearts were also used for studying the action of drugs on ventricular muscle.

The drugs used were benzoylcholine chloride (B.D.H.), acetylcholine chloride, choline chloride, eserine sulphate, *d*-tubocurarine chloride, hexamethonium bromide, nicotine acid tartrate, atropine sulphate, cocaine hydrochloride, and procaine hydrochloride. All were made up in solution in distilled water.

RESULTS

Local anaesthetic activity

Benzoylcholine (in 2 per cent solution) possesses no local anaesthetic property when tested by the standard procedure on the rabbit's cornea. On the shaved skin of the guinea-pig, however, slight activity is observed. The mean value of four determinations showed that benzoylcholine is an eighth as active as procaine by this test.

Action on striated muscle

On the isolated rectus abdominis of the frog, benzoylcholine produces contraction, but it is 5 to 20 times less active than acetylcholine. Its action is little affected by eserine, but tubocurarine (10^{-5}) and procaine (10^{-4}) block the response, just as they do that of acetylcholine. The presence of small doses of benzoylcholine in the bath does not affect the acetylcholine or benzoylcholine response.

When injected into week-old chicks under pentobarbitone or ether anaesthesia, benzoylcholine causes the spastic reaction typical of decamethonium. Benzoylcholine (1 mg./kg.) is more active than acetylcholine by this test and produces rigid extension of the limbs and retraction of the head. The muscle twitch of the cat

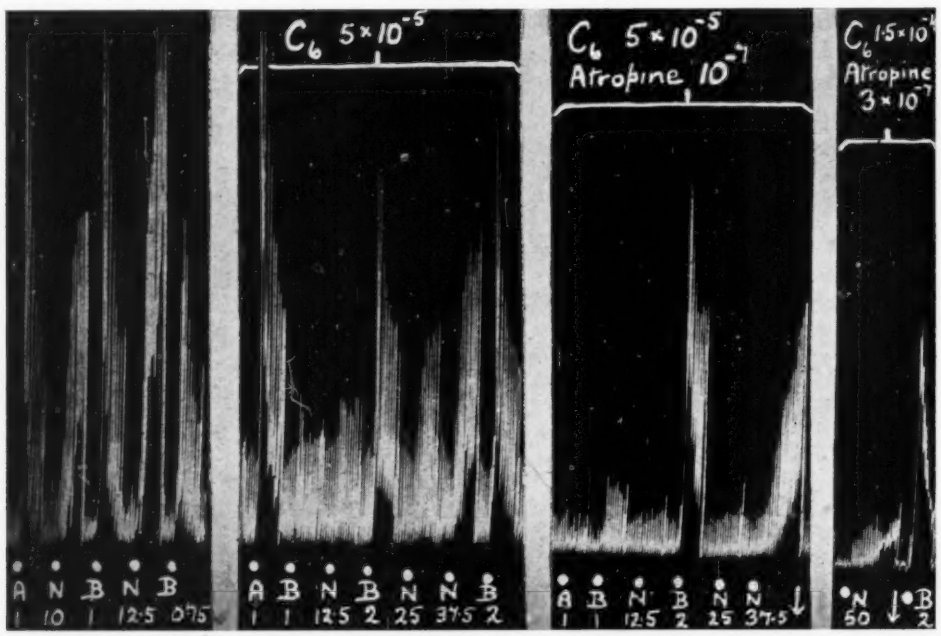

FIG. 1.—Isolated rabbit ileum. Bath 15 ml. Comparison of effects of acetylcholine (A, μg.), nicotine (N, μg.), and benzoylcholine (B, mg.). Hexamethonium (5×10^{-5}) blocks the benzoylcholine and nicotine responses. Larger doses of benzoylcholine are effective in hexamethonium and atropine when nicotine is ineffective. Time in min.

sciatic-gastrocnemius preparation, evoked by single maximal nerve volleys, is depressed by intra-arterially injected benzoylcholine (0.5–2 mg./kg.). This neuromuscular blocking action was also observed in the isolated phrenic nerve-diaphragm preparation of the rat (concentrations of 10^{-4}).

Action on smooth muscle

Isolated intestine.—On the rabbit ileum, benzoylcholine causes stimulation, though it is usually more than 1,000 times less active than acetylcholine (Fig. 1). Hexamethonium (5×10^{-5}) abolishes this stimulation just as it does that of nicotine. The initial stimulation, therefore, is caused by the nicotine-like property of benzoylcholine. Doubling the benzoylcholine dose after hexamethonium (5×10^{-5}) and atropine (10^{-7}) results in recovery of stimulation, whereas increasing the nicotine dose threefold only results in partial recovery (Fig. 1). In the presence of stronger concentrations of hexamethonium and atropine, the double dose of benzoylcholine is effective, whereas increasing the nicotine dose fourfold is ineffective (Fig. 1). Benzoylcholine, therefore, possesses both a hexamethonium-resistant and an atropine-resistant stimulant action.

A similar result was found in the rabbit duodenum. In the presence of hexamethonium an ineffective dose of benzoylcholine prevents both the acetylcholine and the nicotine responses without affecting the benzoylcholine response. Benzoylcholine, therefore, also possesses an atropine-like action.

On the isolated guinea-pig ileum, benzoylcholine is usually more than 4,000 times less active than acetylcholine in producing stimulation. Whereas in this tissue hexamethonium (5×10^{-5}) effectively abolishes the nicotine stimulation, it has little effect on that of benzoylcholine. In the presence of both hexamethonium (5×10^{-5})

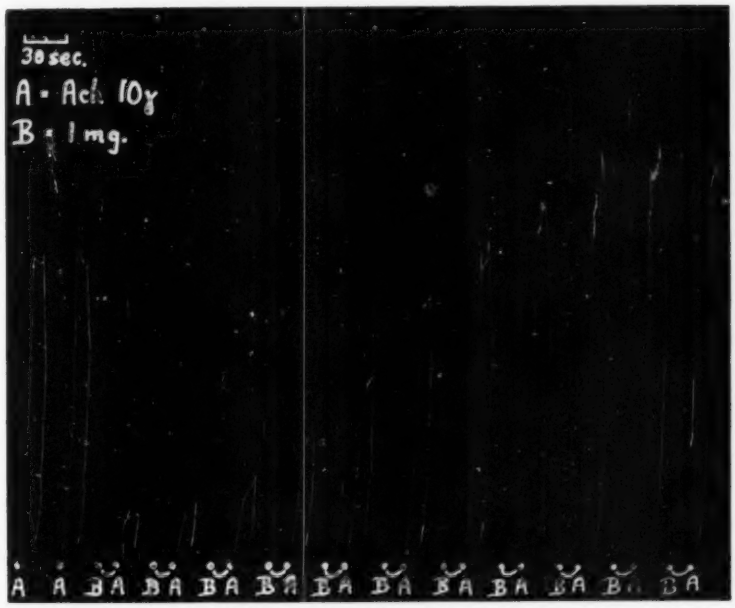

FIG. 2.—Isolated guinea-pig ileum. Bath 15 ml. Effect of benzoylcholine (B, 1 mg.) in the bath on the stimulation produced by acetylcholine (A, 10 μg.). Note that the acetylcholine response is first reduced and then recovers despite the presence ot benzoylcholine. Time in 30 sec.

and atropine (10^{-7}), twice the normal benzoylcholine dose is effective in stimulating the muscle, although increasing the nicotine dose tenfold is almost without effect. When mepyramine (10^{-9}) is added to the perfusing fluid, the action of benzoylcholine is little affected although the histamine response is blocked.

In another series of experiments on guinea-pig ileum, the action of benzoylcholine in blocking the acetylcholine response was further investigated. If the blocking action is repeated many times on the same preparation, an unusual effect is observed (Fig. 2). Slowly and steadily the acetylcholine response recovers despite the fact that effective blocking doses of benzoylcholine are still present in the bath. Very small doses of benzoylcholine ($10^{-7} - 5 \times 10^{-6}$) added before a standard acetylcholine dose do not potentiate its response, but potentiation is usually observed when benzoylcholine is washed out of the bath.

On the peristaltic reflex, large doses of benzoylcholine (2×10^{-4}) almost completely abolish the reflex in a manner similar to that shown by hexamethonium.

Isolated trachea of cat and rabbit.—In these preparations, small doses of benzoylcholine (10^{-5}) potentiate the acetylcholine response, medium doses (10^{-4}) block it, whilst larger doses *per se* (up to 10^{-3}) are without effect (Fig. 3).

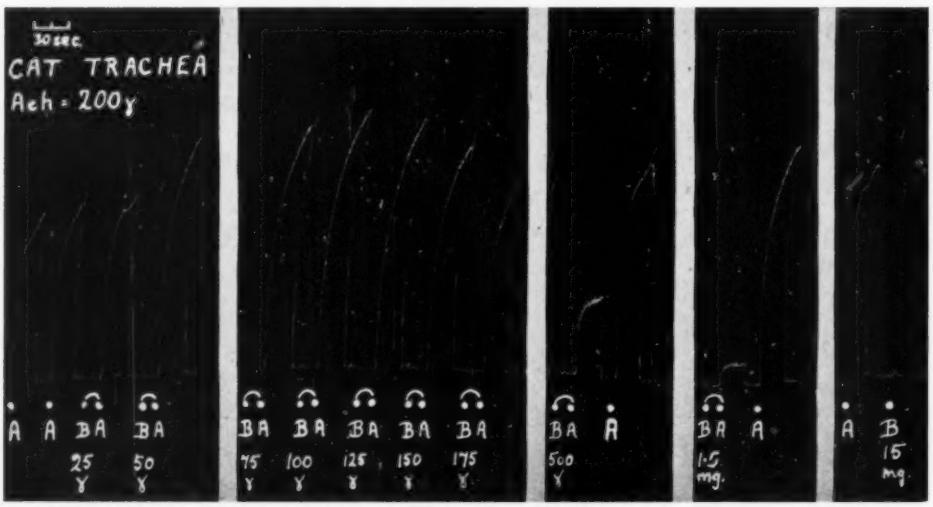

FIG. 3.—Isolated cat trachea. Bath 15 ml. Effect of benzoylcholine (B, μg. or mg.) in the bath on the stimulation produced by acetylcholine (A, 200 μg.). Note that 100 μg. benzoylcholine potentiates, and 500 μg. reduces, the acetylcholine response. Large doses of benzoylcholine (10^{-3}) are without effect in this preparation. Time in 30 sec.

Isolated uterus of guinea-pig.—Benzoylcholine ($2 \times 10^{-4} - 10^{-3}$) causes contraction of the uteri of both pregnant and non-pregnant guinea-pigs, but it is 200–400 times less active than acetylcholine. This muscle-stimulating action of benzoylcholine is not antagonized by concentrations of atropine effective against acetylcholine.

Action on blood pressure

In the freshly anaesthetized cat, an intravenous dose of benzoylcholine (0.1–0.2 mg./kg.) usually causes a transient fall in blood pressure. This fall is abolished by

atropine (1 mg./kg.) so that the effect is due to the muscarine-like property of benzoylcholine. Higher concentrations (0.8–1 mg./kg.) cause a rise in blood pressure. This rise is potentiated by intravenous doses of procaine (2 mg./kg.) or cocaine (1 mg./kg.), but is antagonized by hexamethonium (10 mg./kg.) or adrenalectomy. It represents the nicotine-like property of benzoylcholine. After hexamethonium or adrenalectomy, however, larger doses of benzoylcholine (4–5 mg./kg.) cause a rise in blood pressure, probably resulting from a direct stimulant effect on the muscle in the blood vessels. If the hind-limbs of the rabbit are perfused with Tyrode's solution containing atropine (10^{-6}), the injection of benzoylcholine (1–2 mg.) also causes vasoconstriction.

Action on heart muscle

Benzoylcholine stimulates the isolated rabbit or cat auricles. Small amounts produce a single delayed stimulation lasting about a minute ; larger doses give a biphasic response, initially a rapid transient stimulation followed a few seconds later by a more prolonged stimulation. The initial rapid stimulant action can be blocked by doses of nicotine too small to have a stimulant action themselves, whereas very small doses of benzoylcholine given one minute before effective doses block both stimulant effects. Whereas hexamethonium (5×10^{-6}) prevents stimulation by nicotine, the benzoylcholine response is little affected either by hexamethonium or atropine (Fig. 4). In addition, benzoylcholine ($10^{-5} - 10^{-4}$) blocks the acetylcholine

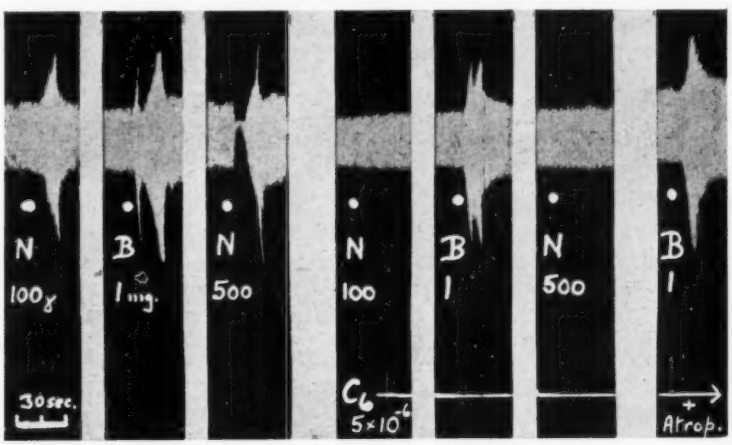

FIG. 4.—Isolated rabbit auricles. Bath 15 ml. Comparison of effects of nicotine (N, μg.) and benzoylcholine (B, mg.) before and after hexamethonium (5×10^{-6}). The stimulant action of benzoylcholine is not affected by hexamethonium or atropine (10^{-7}). Time in 30 sec.

response, and restarts the beat when excess acetylcholine is present. On the isolated perfused rabbit heart (Langendorff preparation) benzoylcholine (100–200 μg.) stimulated the beat, the effects being very similar to those seen in the auricle preparation.

Benzoylcholine increases the amplitude of the beat of the isolated frog heart (Straub preparation). This effect is not due to its nicotine-like property, since nicotine itself slightly inhibits the beat and benzoylcholine stimulates in the presence of nicotine (Fig. 5). A stimulant dose of benzoylcholine (50 μg.) prevents the acetyl-

choline or choline response and starts the ventricle when it has been stopped by acetylcholine or choline. Since this stimulant action of benzoylcholine is unaffected by atropine, it probably represents a direct action on cardiac muscle.

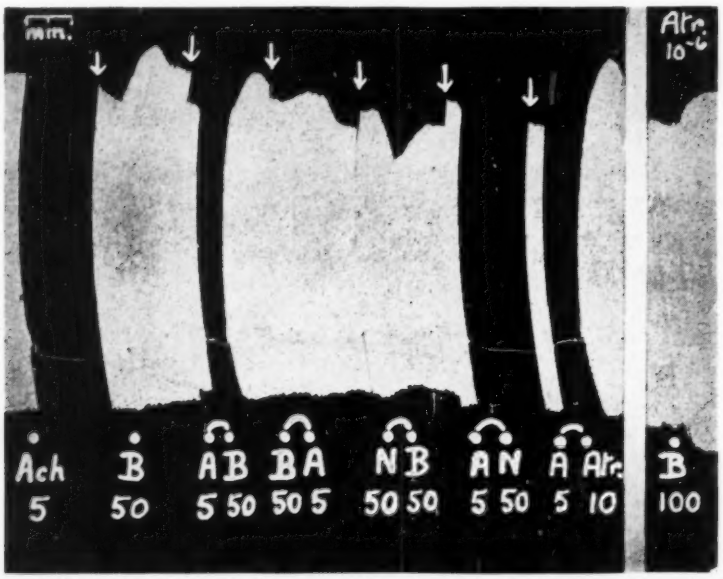

FIG. 5.—Frog heart (Straub preparation). Cannula volume 1 ml. Comparison of effects of acetylcholine (A), benzoylcholine (B), nicotine (N), and atropine (Atr.). Doses in μg. Time in min. Wash at arrows. Note that benzoylcholine stimulates the heart even in the presence of nicotine and atropine. In the presence of excess acetylcholine, benzoylcholine and atropine restart the heart.

DISCUSSION

Benzoylcholine has been widely used in enzyme studies as a substrate for pseudocholinesterase. It is freely soluble in water, and yet its other pharmacological actions appear to have been little studied. It possesses a structural formula which resembles those of many commonly used local anaesthetics, such as procaine and amylocaine, and yet we have found it to be lacking in this property. This is not surprising, since benzoylcholine is a quaternary base and exists in solution as stable cations and anions. On the other hand, most local anaesthetics contain a tertiary amino group, and activity appears to depend upon the concentration of undissociated base (Trevan and Boock, 1927). Bovet and his co-workers (1949) noted that benzoylcholine is a neuromuscular blocking agent in rabbits and is relatively non-toxic (LD50 in rabbits : 150 mg./kg.). We have now shown that it produces neuromuscular block in the rat diaphragm and cat gastrocnemius and paralysis in chicks. In chicks it produces spasticity so that benzoylcholine probably causes depolarization of the endplate region of the muscle fibre, like that produced by decamethonium or succinylcholine. It does not act like curare, for in the frog rectus muscle it has no action on the acetylcholine response but itself causes stimulation, which is blocked by curare and procaine. Its action is little affected by eserine.

On smooth muscle, benzoylcholine appears to exert at least three distinct actions. In small doses in the trachea preparations, it potentiates the acetylcholine response,

possibly by inhibition of the cholinesterase ; in medium doses in all preparations, it blocks the acetylcholine response, possibly by attaching itself to the same receptors ; and in larger doses, it stimulates most forms of smooth muscle, in part through a direct stimulant action.

The weak muscarine-like property of benzoylcholine is best shown on the blood pressure of a freshly anaesthetized animal. Repeated doses, however, usually result in the disappearance of the hypotensive response and the appearance of hypertension (nicotine-like action).

There is no doubt that benzoylcholine directly stimulates auricular and ventricular cardiac muscle, and it is the only choline derivative so far studied that will stimulate the heart in the presence of acetylcholine. The blocking action of benzoylcholine on acetylcholine is possibly the result of competitive antagonism, as was suggested in the smooth-muscle experiments, but further work on rabbit auricles suggests that at least five times as many molecules of benzoylcholine must be present for the usual acetylcholine response to be overcome. Benzoylcholine is known to be broken down in the body into benzoic acid and choline, but a solution of equimolar amounts of benzoic acid and choline does not exert the effects of benzoylcholine ; in fact, the mixture behaves like a solution of choline, so that it must be the benzoylcholine molecule itself which is responsible for the observed effects.

All the results suggest that benzoylcholine may be a useful tool for the study of the mechanism by which acetylcholine exerts its characteristic actions.

Summary

1. Benzoylcholine possesses strong nicotine-like properties and weak muscarine-like properties.

2. In addition, benzoylcholine has a direct stimulant action on gut and heart ; this action is unaffected by atropine.

3. Benzoylcholine restarts the frog heart and rabbit auricles after they have been stopped by excess of acetylcholine.

4. Benzoylcholine blocks the acetylcholine response on gut, trachea, and heart.

5. On the rabbit auricles, small ineffective doses of benzoylcholine block the response of effective doses of benzoylcholine.

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THE PHARMACOLOGICAL ACTIONS OF 6-METHYLADRENALINE

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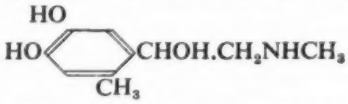
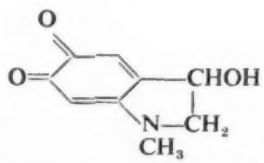
N-methyl-3 : 4-dihydroxy-6-methylphenylethanolamine or 6-methyladrenaline (I) was recently prepared by Dr. E. J. Forbes, under Sir Robert Robinson's direction, and I was given the opportunity of studying its pharmacological properties. The



(I)

main interest of this compound arises from its inability to undergo one of the chemical reactions postulated as responsible for the inactivation of adrenaline *in vivo*.

Adrenaline may be inactivated in the body in several ways : by the oxidative action of amine oxidase ; by the formation of adrenochrome (II) ; by the oxidative action of polyphenol oxidase ; and by conjugation as a catechol acid sulphate or glycuronide.



(II)

The last method is known to occur when adrenaline is administered orally (Richter, 1940 ; Beyer and Shapiro, 1945), but it may be only a subsidiary path of inactivation for physiologically liberated adrenaline. Inactivation by polyphenol oxidase has not yet been demonstrated *in vivo*.

The function of amine oxidase as an inactivating mechanism has been questioned by Bacq (1949), who thinks that adrenochrome formation is the main route by which adrenaline is inactivated. 6-Methyladrenaline, owing to the methyl group, is incapable of forming an adrenochrome-like compound, and Dr. H. Blaschko, who compared the oxidation of 6-methyladrenaline with that of adrenaline, found that potassium iodate did not oxidize it to an iodochrome type of compound. Consequently 6-methyladrenaline, simply because it is incapable of forming an adrenochrome-like compound, is a substance of considerable pharmacological interest.

Experimental Observations

Preparation of solution.—When dissolved in water in the ordinary way, 6-methyladrenaline (base) rapidly changed in colour, and steps were therefore taken to prepare

a stable solution before each experiment. Saline was first boiled to expel dissolved air, and was covered with liquid paraffin. A weighed portion of 6-methyladrenaline was dissolved on a watch glass in 0.1 N-HCl, and then made up to the required volume in a cylinder with the boiled saline. The solution was at once covered with liquid paraffin. Dilutions of this solution were also made with boiled saline. In many experiments, e.g. on the isolated heart, each injection was freshly prepared.

Action on the blood pressure.—When examined on the spinal cat the pressor effect of methyladrenaline resembled that of adrenaline or of noradrenaline, though when the doses produced the same rise of blood pressure the effect of methyladrenaline was more prolonged than that of either. This difference is shown in Fig. 1, which also shows that methyladrenaline caused a very prolonged contraction of the nictitating membrane. In eight experiments on the spinal cat, the dose of methyladrenaline causing the same rise of blood pressure as adrenaline varied from 10 to 50 times the dose of adrenaline, the mean figure being 21.

In the cat anaesthetized with chloralose, 6-methyladrenaline appeared to be more potent than in the spinal cat, the equipressor dose in four experiments ranging from 5 to 16 times the dose of adrenaline, the mean figure being 9.

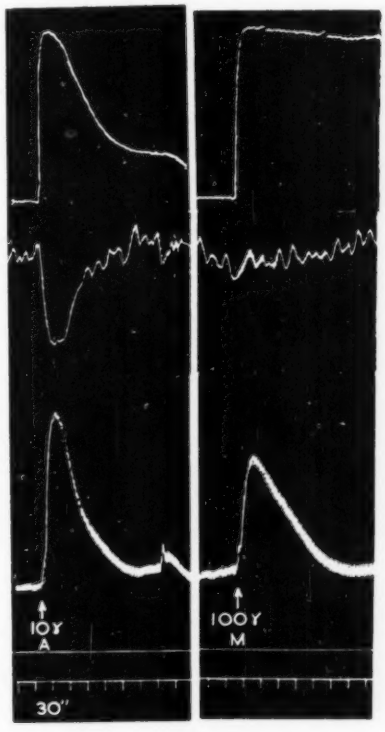


FIG. 1

FIG. 1.—Spinal cat. Top record, normal nictitating membrane; middle record, uterus; bottom record, arterial blood pressure. Injections of 10 μg. adrenaline (A), and of 100 μg. 6-methyladrenaline (M). Note that methyladrenaline caused a more prolonged contraction of the nictitating membrane than adrenaline, but had no action on the uterus.

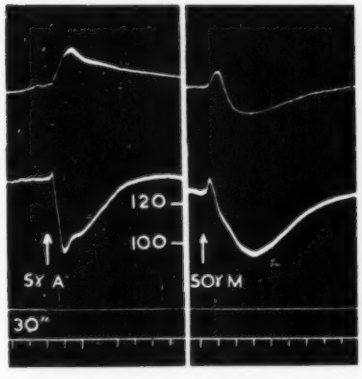


FIG. 2

FIG. 2.—Cat under ether, vagi cut. Upper record, volume of left hindleg, in which the sciatic nerve had been divided 8 days previously. Lower record, arterial blood pressure. Injections of 5 μg. adrenaline (A), and 50 μg. 6-methyladrenaline (M). Note that both injections caused a fall in blood pressure, but, whereas the hindleg was dilated when adrenaline was injected, it was constricted when 6-methyladrenaline was injected.

In occasional experiments it was observed that 6-methyladrenaline produced, after an initial rise, a fall of blood pressure as its main effect. With a larger dose the ordinary rise reappeared, but was followed by a fall.

In cats anaesthetized with ether, when the vagi were cut, this fall was commonly seen and resembled the fall produced by the injection of small amounts of adrenaline. In four cats plethysmograph records of the left hindleg were obtained, the sciatic nerve having been cut 7–9 days before in an aseptic operation. These cats were also examined under ether with the vagi cut, and Fig. 2 illustrates that 6-methyladrenaline did not cause dilatation of the hindleg as did adrenaline. Both substances lowered the blood pressure. Adrenaline causes the fall because of the dilatation of the muscle vessels ; 6-methyladrenaline does not cause a fall in this way, and the decline in limb volume might appear to be passive. This, however, is not so, because in some experiments the same fall of limb volume accompanied a rise in blood pressure. The action of 6-methyladrenaline on the hindleg vessels was therefore constrictor.

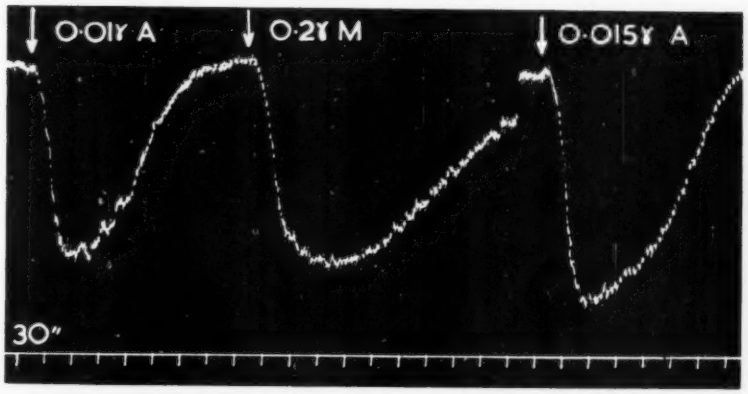
Another possible cause of the fall of blood pressure produced by 6-methyladrenaline was a depression of ganglionic transmission of vasoconstrictor impulses to the blood vessels. Adrenaline is known to have such an action. Two experiments were therefore performed in which the cervical sympathetic chain was divided and laid upon electrodes in a cat anaesthetized with chloralose. The chain was stimulated so as to produce a large and maintained contraction of the nictitating membrane. 6-Methyladrenaline (0.05–0.5 mg.) was then injected intravenously, but it had no effect on the contraction such as would have been caused by a small dose of hexamethonium.

Action on blood vessels.—The action on blood vessels was studied in the isolated rabbit ear perfused through the central artery with Locke's solution. A record was made of changes in outflow using Stephenson's recorder (1948). 6-Methyladrenaline had a similar constrictor action to adrenaline, though as a rule it was more prolonged, as shown in Fig. 3*a*. The dose which produced the same constriction was, in ten experiments, from 10 to 20 times the dose of adrenaline, the mean figure being 12.

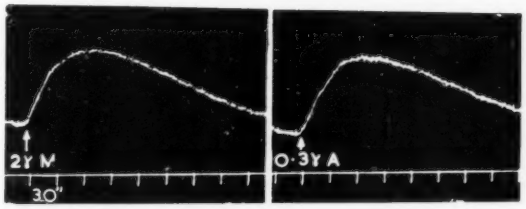
When benzylimidazoline (Priscol) was added to the perfusing fluid in a concentration 2×10^{-4}, the action of 6-methyladrenaline, like that of adrenaline, was reversed, as shown in Fig. 3*b*.

Action on the heart.—The action of sympathomimetic amines on the heart is known to vary ; thus ephedrine has rarely any effect on the rabbit heart, but on the cat heart its stimulant action is regularly seen. The experiments were therefore carried out on the isolated heart of the cat perfused by the Langendorff method. The action was always weak and very variable. In fifteen experiments, the rate was sometimes increased with the first injection of 6-methyladrenaline and then unaffected by subsequent injections. In some experiments the injection of 6 μg. caused an increase, while the injection of larger amounts, 10 or 20 μg., had no effect ; subsequently the smaller amount (6 μg.) again increased the rate. In other experiments the rate was unaffected by all amounts injected from 2–20 μg.

The variation is best illustrated by the fact that, when 4 μg. 6-methyladrenaline was injected on twelve occasions in twelve different preparations, it failed to modify the rate in six trials, while it increased the rate by 11, 14, and 33 per cent respec-



(a)

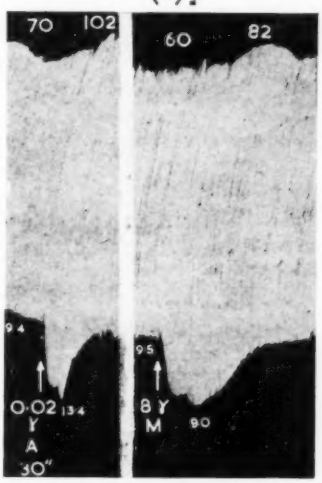


(b)

FIG. 3.—Record of outflow from vessels of rabbit ear perfused with Locke's solution. (*a*) 0.2 μg. 6-methyladrenaline (M) caused a constriction similar in size (but longer in duration) to that caused by 0.01 μg. adrenaline (A). (*b*) When the perfusion fluid contained benzylimidazoline (0.2×10^{-3}), the effect of both substances was reversed. 2 μg. methyladrenaline had a similar effect to that of 0.3 μg. adrenaline.

tively in three trials; in the remaining three trials there was a slight increase in the rate (2, 2, and 6 per cent). When 10 μg. 6-methyladrenaline was injected, it failed to affect the rate in six out of seven trials. When an increase of rate was obtained and compared with that produced by adrenaline, the result, illustrated in Fig. 4*a*, showed that 6-methyladrenaline was from 200 to 1,200 times weaker than adrenaline, though in one experiment a figure of 30 times was obtained.

(a)



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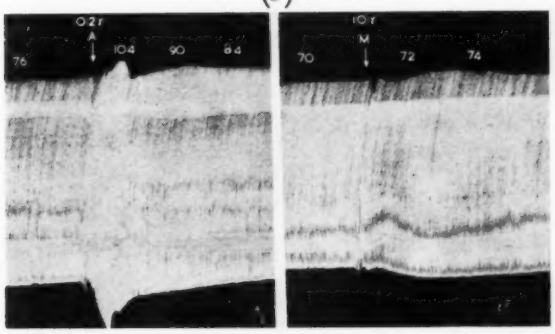

FIG. 4.—Isolated cat heart perfused with Locke's solution by Langendorff's method. Figures above the record are heart rate per min. Figures below are outflow in ml./min. (*a*) In this preparation the injection of 8 μg. 6-methyladrenaline (M) caused an increase in amplitude and rate similar to that caused by 0.02 μg. adrenaline (A). (*b*) In this preparation, 10 μg. 6-methyladrenaline (M) had a very slight effect on the amplitude and rate.

The effect on the amplitude of contraction was more regular than the effect on the rate, and it was more prolonged. The result shown in Fig. 4*b* was, however, seen as often as that in Fig. 4*a*. The mean increases produced by the amounts 2, 4, and 6 μg. were 9, 11, and 30 per cent respectively, but larger doses, 10 and 20 μg., had less effect, increasing the amplitude by 17 and 16 per cent respectively. Even so, in relation to the effect of adrenaline, the effect of 6-methyladrenaline on the amplitude was very weak, and in no way corresponded to the relative vasoconstrictor action.

Action on isolated auricles.—When added to a bath containing the isolated auricles of the rabbit, 6-methyladrenaline in concentrations up to 0.2×10^{-5} had no action. On auricles stimulated electrically by the method described by Dawes (1946), 6-methyladrenaline acted differently from adrenaline in that it failed to increase the maximum rate at which the auricles would follow an applied stimulus. The action of adrenaline has been described by Elío (1947). 6-Methyladrenaline, on the contrary, depressed the maximum rate, as shown in Fig. 5. The results of three experiments are given in Table I ; similar results were obtained in five other experiments.

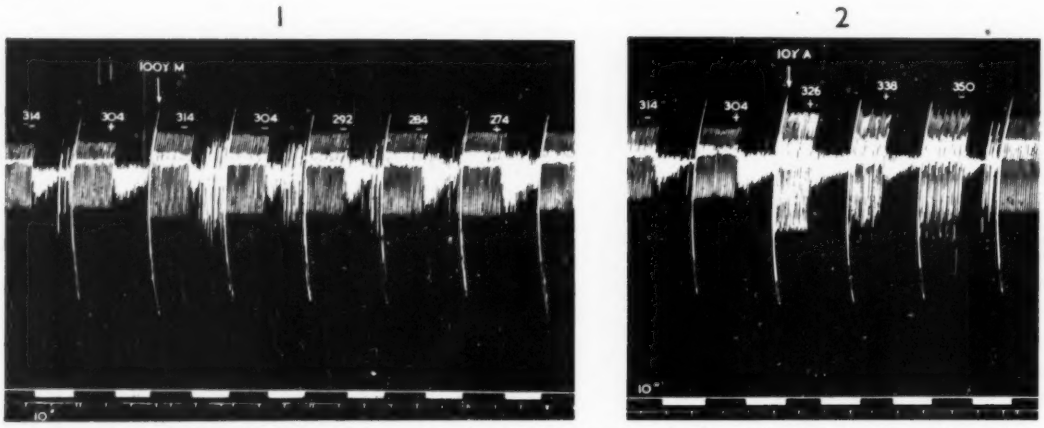

FIG. 5.—Record of isolated auricles arranged to be driven electrically. The first tracing shows that 100 μg. 6-methyladrenaline reduced the maximum rate at which the auricles would follow an applied stimulus from 304 to 274 per min. The second tracing shows that 10 μg. adrenaline increased the maximum rate from 304 to 338 per min.

Action on coronary vessels.—The effect of 6-methyladrenaline on the outflow in the experiments made with the Langendorff preparation showed that in four out of seven experiments it increased the coronary flow as did adrenaline. In the other three experiments 6-methyladrenaline decreased the coronary flow ; in one of these adrenaline also decreased the coronary flow (compare Kordik, 1951), in one adrenaline had no effect, and in one adrenaline increased the flow.

Nictitating membrane and pupil.—Reference has already been made to the effect on the nictitating membrane, which, as shown in Fig. 1, is very prolonged. In seven experiments the mean figure for the amount of 6-methyladrenaline producing the same height of contraction as a given amount of adrenaline was seven times as great. In four cats the superior cervical ganglion was removed on the right side, and 7–9 days later the contractions of the normal and denervated membranes were recorded

TABLE I

EFFECT OF ADRENALINE AND 6-METHYLADRENALINE ON MAXIMUM RATE OF DRIVEN AURICLES OF THE RABBIT

Bath 100 ml.

Exp.	Adrenaline		6-Methyladrenaline	
	Dose μg.	Effect (%)	Dose μg.	Effect (%)
1	10	+17.3	100	−15.3
			200	−12.1
2	10	+21	100	−8.9
			200	−8.2
			400	−12.6
3	10	+9.8	100	−17.1
			200	−28.6

in the spinal preparation. Fig. 6 shows side by side the effects of injecting adrenaline and 6-methyladrenaline. The denervated membrane contracted more in response to 6-methyladrenaline than did the normal membrane, but the difference was much less than the difference for adrenaline. In both membranes the effect of 6-methyladrenaline was more prolonged than that of adrenaline.

On the pupil the action of 6-methyladrenaline was weak ; in two cats in which eight and seven observations were respectively made, the amounts of 6-methyladrenaline having the same effect by intravenous injection as a given amount of adrenaline were 60 and 62.5 times as great.

Intestinal balloon.—A balloon was prepared by attaching a rubber fingerstall to a catheter and filling it with water ; the balloon was then inserted into the proximal end of the small intestine of a cat under chloralose anaesthesia. Injections of 6-methyladrenaline and of adrenaline were given when the pressure changes in the balloon shown by a piston recorder were sufficiently regular. Seven experiments were performed, and in the last experiment both adrenal glands were excluded from the circulation.

In causing inhibition of intestinal movement, 6-methyladrenaline was more powerful, relative to adrenaline, than in any other action. Fig. 7 shows that 10 μg. 6-methyladrenaline caused a more prolonged inhibition than either 2 μg. or 4 μg. adrenaline. The mean figure from the seven experiments for the amount of 6-methyladrenaline producing the same inhibition as a given amount of adrenaline was 2.55 times as great.

Peristalsis in guinea-pig ileum.—Observations were also made on the Trendelenburg preparation of the guinea-pig ileum *in vitro*, in which the peristaltic waves induced by raising the pressure in the lumen of the gut were recorded. Doses of adrenaline and of 6-methyladrenaline just sufficient to inhibit peristalsis were determined. In four experiments the mean figure for 6-methyladrenaline was 6.5 times as great as for adrenaline.

Pendular movements.—On the isolated duodenum of the cat and of the rabbit the inhibitory action of methyladrenaline was very much weaker. The mean figure for an amount of 6-methyladrenaline having an equivalent action to an amount of adrenaline was about 200 times greater both for cat and for rabbit duodenum.

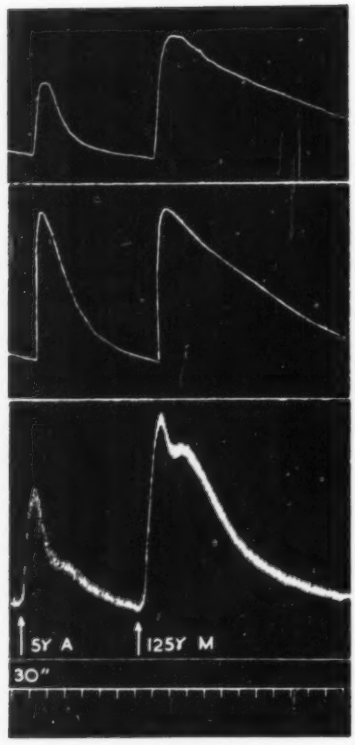

FIG. 6

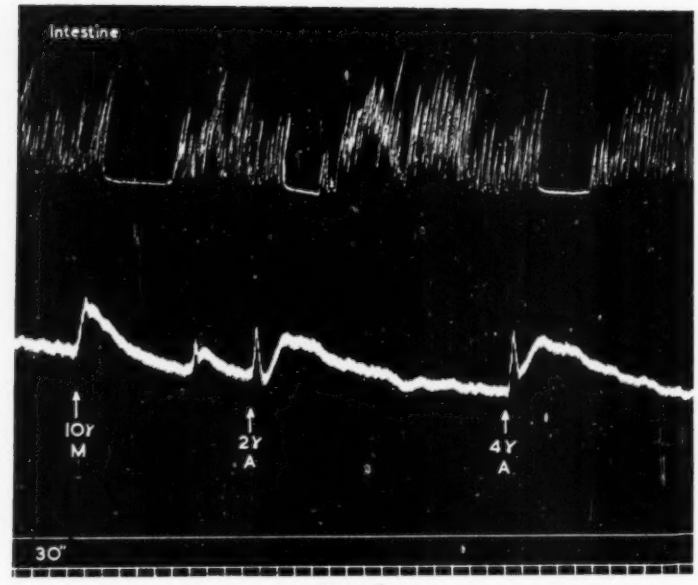

FIG. 7

FIG. 6.—Spinal cat. Comparison of the action of 5 μg. adrenaline (A) and of 125 μg. 6-methyladrenaline (M) on the normal nictitating membrane (top tracing), on the denervated nictitating membrane (middle tracing), and on the blood pressure (bottom tracing). Both substances had about the same effect on the denervated membrane, but with each substance this was greater than the effect on the normally innervated membrane.

FIG. 7.—Cat under chloralose. Upper tracing, record of pressure changes in intestinal balloon inserted in the duodenum. Lower tracing, arterial blood pressure. Note that 10 μg. 6-methyladrenaline (M) caused an inhibition greater than that caused by 2 μg. adrenaline (A) or than that caused by 4 μg. adrenaline.

Blood sugar.—Experiments were carried out to compare the action of adrenaline and of 6-methyladrenaline on the blood sugar. After preliminary observations were made, it was decided to test the effect of 60 μg. adrenaline and of 1 mg. 6-methyladrenaline. Each substance was injected subcutaneously in the dose stated into each of two rabbits, as shown in Table II. The rabbits, which weighed 2.4 and 2.1

TABLE II

RISE OF BLOOD SUGAR IN THE RABBIT PRODUCED BY ADRENALINE AND 6-METHYLADRENALINE

Drug and dose	Percentage hyperglycaemia in Rabbit 1	Rabbit 2
0.06 mg. adrenaline ..	70, 25.6, 57.7 (Mean, 51.1)	77.5, 65 (Mean, 71.2)
1 mg. 6-methyladrenaline ..	45.5, 57, 57 (Mean, 53)	47, 77 (Mean, 62)

kg. respectively, were kept without food overnight. A sample of blood was taken before the injection was given, and again at hourly intervals for 5 hr. The blood sugar was determined by precipitating the proteins according to Somogyi's method, and estimating the sugar by the method of Hagedorn-Jensen. The mean blood sugar values for the five samples after injection were calculated, and the rises of blood sugar were expressed as percentages of the initial values. The percentage hyperglycaemia in the different experiments is shown in Table II. The mean figure for the percentage hyperglycaemia caused by 0.06 mg. adrenaline in the two rabbits is 61 as compared with 58 for 1 mg. 6-methyladrenaline. Thus 6-methyladrenaline has a similar action in 16 times the dose.

Rabbit uterus.—The action of adrenaline on the isolated rabbit uterus is motor, and 6-methyladrenaline was found to have a similar motor effect. A comparison with adrenaline was made on one horn of the uterus of five rabbits, and it was found that the mean figure for the dose exerting a given effect was 23 times as great as the dose of adrenaline. The five figures varied from 3.5 to 33.3, though four were between 20 and 33. The effect of 6-methyladrenaline was abolished like that of adrenaline in the presence of 10^{-6} ergotamine tartrate.

Spleen.—Observations were made on the spleen with its natural circulation by withdrawing it through an incision in the side of the cat and enclosing it in a plethysmograph ; the volume changes were recorded by a piston recorder. One cat was anaesthetized with chloralose ; the rest were spinal preparations. In the cat under chloralose and in one spinal cat, the doses of 6-methyladrenaline causing the same contraction as a dose of adrenaline were respectively 5 and 10 times as great. In the other three spinal cats, however, the doses were 50, 100, and 200 times as great. The effect of 6-methyladrenaline was of greater duration than that of adrenaline. Thus the mean figure of the different experiments was 73, though the very wide variation detracts from the value of this mean.

Cat uterus.—The cat uterus was used *in situ* in four experiments, and isolated in a bath in two experiments. In none of the experiments was 6-methyladrenaline observed to cause inhibition, though in each the effect of small amounts of adrenaline was inhibitory.

Bronchioles.—The action on the bronchioles was studied using the Konzett–Roessler apparatus in the guinea-pig anaesthetized with urethane. The inhibitory effect was very weak here also. Acetylcholine was injected in a dose of 8–10 μg.; this caused a transient bronchoconstriction. When adrenaline or 6-methyladrenaline was injected 45 sec. before the acetylcholine, the bronchoconstriction was less. In three experiments the amounts of 6-methyladrenaline required to produce the same effect as adrenaline were respectively 50, 80, and 200 times as great.

Skeletal muscle.—Experiments were carried out on the sciatic-gastrocnemius preparation of the cat anaesthetized with chloralose. The method used was that described by Bülbring and Burn (1942), in which the detached tendo Achillis is arranged to pull on a tension lever, the sciatic nerve being stimulated at regular intervals by maximal single shocks. When contractions of regular height were obtained, an injection of neostigmine (usually 10 μg.) was made into the central cut end of the external iliac artery of the opposite side, the injection being made almost into the bifurcation of the aorta so that the fluid was carried to the stimulated

muscle with little dilution. The injection caused a rise of tension as shown in Fig. 8, and, when this rise had reached its peak and was declining, an injection of 6-methyladrenaline or of adrenaline was made. Adrenaline in amounts of 10–20 μg. usually increased the tension again. 6-Methyladrenaline was without effect in amounts from 0.1 to 0.5 mg., but, in two experiments out of five, 1 mg. 6-methyladrenaline had a small effect, as shown in Fig. 8. Thus 6-methyladrenaline had very little action compared with adrenaline.

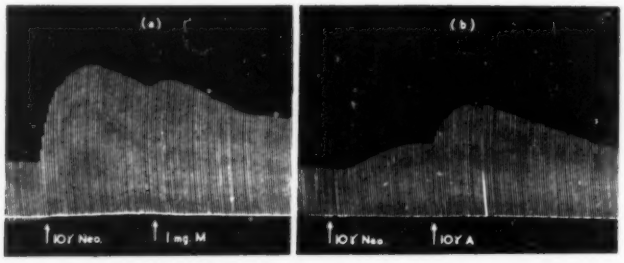

FIG. 8.—Record of twitch tension in gastrocnemius muscle of cat under chloralose caused by single shocks applied to the sciatic nerve. In (*a*) the injection of 10 μg. neostigmine (Neo) given into the bifurcation of the aorta caused an increase in the tension, and, as this declined, the injection of 1 mg. 6-methyladrenaline (M) by the same route caused a slight further increase. In (*b*) the augmentation due to 10 μg. adrenaline (A), again injected after 10 μg. neostigmine, is shown.

Bülbring preparation.—Experiments were also carried out on the Bülbring preparation of the phrenic nerve-diaphragm of the rat. In this also the effect of adrenaline was tested after the addition of neostigmine in a concentration 2.5×10^{-9}, which produced a steady increase in the contractions. A concentration of 4×10^{-7} adrenaline, applied after washing out the neostigmine, caused a prompt increase in the contractions. When 6-methyladrenaline was tested, no augmentor effect was recorded even with doses of 2 mg.

DISCUSSION

The action of 6-methyladrenaline differs from that of adrenaline chiefly in affecting the isolated heart so little. In addition it lacks an inhibitory action on the non-pregnant uterus of the cat, and is weak as a bronchodilator; on the other hand, it has a fairly powerful inhibitory action on the intestine. Since it possesses most of the other actions of adrenaline, it might be concluded that its effect is exerted primarily on the receptors which Ahlquist (1948) has called "α" receptors, and that it has little or no action on "β" receptors. The action on blood vessels, and in various other tissues where it exerts a motor or stimulant action, is frequently of the order of 1/12th that of *l*-adrenaline. Blaschko (1950) has pointed out that both *d*-adrenaline and epinine have about 1/12th of the action of *l*-adrenaline also, and that this results from the less firm combination with the receptors they are able to effect. He suggests that *l*-adrenaline has three points of anchorage, whereas *d*-adrenaline and epinine have only two. A similar explanation may hold for 6-methyladrenaline.

The suggestion that 6-methyladrenaline acts only on "α" receptors and not on "β" receptors is, however, not supported by the fact that 6-methyladrenaline

possesses vasodilator effects ; these are seen in the isolated vessels of the rabbit ear, when perfused with Lock's solution containing benzylimidazoline, and as a fall in blood pressure when 6-methyladrenaline is injected into a cat, anaesthetized with ether, in which the vagi have been cut.

Since 6-methyladrenaline cannot form an adrenochrome-like compound, its metabolic fate must lie in a different direction. An attempt to discover whether it was a substrate of amine oxidase was unsuccessful, since the uptake of oxygen in the absence of amine oxidase was too great. However, the prolonged action it exerts on the nictitating membrane, whether denervated or not, suggests that it is removed from its site of action by diffusion rather than by enzymic destruction, though when the dose is so large this may be a wrong conclusion. The contrast between 6-methyladrenaline and adrenaline is well shown in Fig. 6. This figure shows, moreover, that 6-methyladrenaline causes a rather greater contraction of the denervated membrane than of the normal membrane, though the difference is very much less than with adrenaline. The size of the difference may again depend on the dose.

While 6-methyladrenaline is unstable *in vitro,* it may be less so in the body, and it might be an interesting compound from the point of view of therapeutics, since it has so little action on the heart. Many adrenaline derivatives have a limited usefulness because of the increase in the rate and force of the heart beat which they produce. A derivative which had little or no cardiac action would therefore be very useful. Unfortunately, 6-methyladrenaline has a very weak dilator action on the bronchioles. If this action could be intensified by altering the substituent attached to the N-atom, and if the compound could be rendered more stable, a useful therapeutic agent might result.

Summary

1. The pharmacological properties of N-methyl-3 : 4-dihydroxy-6-methylphenyl-ethanolamine (6-methyladrenaline) have been examined. The compound is of interest because it cannot form an adrenochrome-like compound and the blocking of this pathway of degradation may modify its properties.

2. 6-Methyladrenaline can be said briefly to possess the stimulant actions of adrenaline, with the exception of that on the heart, and to possess also the inhibitory action on the intestine. It lacks the inhibitory action on the uterus of the cat, which is so characteristic of adrenaline, and it is much weaker than adrenaline as a bronchodilator.

3. The structures on which the action of 6-methyladrenaline has been examined can be divided into three groups.

On the blood pressure, on isolated blood vessels, on the nictitating membrane, in producing hyperglycaemia, on the rabbit uterus, and on the intestine, the action of 6-methyladrenaline is similar to that of adrenaline, but is from 2.5 to 23 times weaker according to the organ.

On the cat uterus it has no action, and on the heart a very weak and inconstant action.

On the spleen, on the iris of the cat, and on the bronchioles, the action is much weaker than in the first group.

I am deeply indebted to Professor J. H. Burn for his guidance and encouragement at all the stages of this work.

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